

University of Dundee

DOCTOR OF PHILOSOPHY

Molecular and Genetic Mechanisms in Atopic Eczema

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Molecular and Genetic Mechanisms in Atopic Eczema

Sara Judith Brown

Thesis submitted for PhD by publication

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Declaration

“The candidate, Sara J Brown, is the author of this thesis and all references cited have been consulted by the candidate. The work of which the thesis is a record has been done by the candidate, and it has not been previously accepted for a higher degree. Where collaborative research is included in this thesis, the nature and extent of the candidate's individual contribution is defined in Appendix I.”

Signed:

Sara J Brown

01 May 2020

Abbreviations

AhR	Aryl hydrocarbon receptor
<i>AKT1</i>	Gene encoding serine/threonine-protein kinase 1
<i>ANXA6</i>	Gene encoding annexin A6
BEEP	Clinical trial of barrier enhancement for eczema prevention
<i>BRCA2</i>	Gene encoding BRCA2 (breast cancer type 2 susceptibility protein)
ALSPAC	Avon Longitudinal Study of Parents and Children
CAD	Chronic actinic dermatitis
<i>CARD11</i>	Gene encoding Caspase Recruitment Domain Family Member 11
Cas9	CRISPR-associated protein 9
CRISPR	Clustered regularly interspaced palindromic repeat
DRS	Direct (single molecule) RNA sequencing
<i>DSG1</i>	Gene encoding desmoglein 1
<i>DSP</i>	Gene encoding desmoplakin
EDC	Epidermal differentiation complex
<i>EMSY</i>	Gene encoding EMSY
FDR	False discovery rate
<i>FLG</i>	Gene encoding filaggrin
<i>FLG-AS1</i>	Gene encoding filaggrin antisense transcript 1, a long noncoding RNA
GO	Gene ontology
GWAS	Genome-wide association study / studies
HLA	Human leukocyte antigen
IgE	Immunoglobulin E
IL	Interleukin
IV	Ichthyosis vulgaris
LEKTI	Lympho-epithelial Kazal-type-related inhibitor
LLCA	Longitudinal latent class analysis
<i>LRRC32</i>	Gene encoding leucine-rich repeat containing 32
MED	Minimum erythematous dose
MHC	Major histocompatibility complex
MR	Mendelian randomisation
NICE	National Institute for Health and Care Excellence
OMIM	Online Mendelian inheritance in Man

OR	Odds ratio
PIAMA	Prevention and Incidence of Asthma and Mite Allergy
PCR	Polymerase chain reaction
PRIDE	Proteomics identification database (https://www.ebi.ac.uk/pride/)
<i>PRKRA</i>	Gene encoding protein activator of interferon-induced protein kinase
<i>RAD50</i>	Gene encoding RAD50 double strand break repair protein
RISC	RNA-induced silencing complex
RNAi	RNA inhibition
SAM	Syndrome of severe dermatitis, multiple allergies and metabolic wasting
SIGN	Scottish Intercollegiate Guidelines Network
siRNA	Small interfering RNA
shRNA	Small hairpin RNA
SNP	Single nucleotide polymorphism
<i>SPINK5</i>	Gene encoding serine peptidase inhibitor Kazal-type 5
TGF	Transforming growth factor
TEWL	Transepidermal water loss
<i>TNIP1</i>	Gene encoding TNFAIP3- interacting protein 1
UCA	Urocanic acid
UV	Ultraviolet radiation
<i>WAS</i>	Gene encoding Wiskott-Aldrich syndrome protein
WES	Whole exome sequencing

Abstract

Atopic eczema is a common, complex trait that is highly heritable. This thesis describes a body of research over 5 years, from 2014 to 2019, highlighting six key publications. These are described on a background of intense activity within the field of atopic eczema genetic research, stimulated by the discovery, in 2006, of loss-of-function mutations in the gene encoding filaggrin (*FLG*). *FLG* null mutations play a major role in eczema and other atopic diseases and my work has built on this seminal discovery. I described, for the first time, transcriptomic features of *FLG* haploinsufficiency (Cole *et al. J Allergy Clin Immunol* 2014). I tested the effect of *FLG* null mutations on the skin's response to ultraviolet-induced erythema *in vivo* (Forbes *et al. J Allergy Clin Immunol* 2016). I optimised a well-established organoid model, using primary human keratinocytes and fibroblasts to recapitulate aspects of human skin for functional testing of specific genetic effects. Quantitative global proteomic analysis of the organoid model with *FLG* knockdown has replicated findings from previous transcriptome analyses and showed evidence of keratinocyte-immune cell cross-talk and disordered axon guidance (Elias *et al. Wellcome Open Research* 2019). Detailed analysis of the organoid model with knockdown of the transcriptional repressor EMSY has shown, for the first time, that this protein is a master-regulator of multiple aspects of skin barrier formation (Elias *et al. J Allergy Clin Immunol* 2019). Taking a statistical approach driven by a clinical question, longitudinal latent class analysis applied to two independent populations identified distinct subgroups of eczema (Paternoster *et al. J Allergy Clin Immunol* 2018) in which the most long-lasting phenotypes show the greatest genetic risk, including *FLG* null genotype. Finally, leveraging the similarities and differences between eczema and psoriasis, a genome-wide comparative analysis identified opposing genetic mechanisms in these common inflammatory skin diseases (Baurecht *et al. Am J Hum Genet* 2015) providing a theoretical rationale for therapies designed to rebalance the skin's tendency to atopic *versus* psoriatic inflammation. Together this body of work has substantially increased understanding of genetic mechanisms in atopic skin, with important clinical applications and opportunities for much-needed therapeutic development.

CHAPTER ONE: Introduction

Atopic eczema

Nomenclature

Atopic eczema is a term used synonymously with ‘atopic dermatitis’ and ‘eczema’;¹ the nomenclature has continued to be a topic for debate in the field over several decades.²⁻¹⁷ For the purposes of this thesis I will use the term ‘atopic eczema’ when there is evidence of some knowledge relating to an atopic (IgE-mediated) immune response and ‘eczema’ as an umbrella term, in keeping with the World Allergy Organisation nomenclature,¹ where specific atopic features are not clearly defined.

Clinical features

Atopic eczema is an itchy inflammatory skin condition associated with significant morbidity.^{11,15,18} It is characterised by skin barrier impairment, cutaneous and systemic inflammation.¹⁸

Eczema is highly prevalent, affecting up to 25% of school-aged children (with a range from 0.2% to 24.6% worldwide)^{19,20} and up to 10% of adults.²¹ It characteristically presents in childhood and 70% of cases show onset before 5 years of age.¹⁵ However eczema follows a chronic relapsing and remitting course with over 10% of cases persisting into adult life or recurring in adulthood.¹⁵ A subset of eczema cases develop for the first time in adult life^{22,23} and it is not clear whether they may be atopic eczema, irritant contact or allergic dermatitis, or a combination of these disorders. Approximately 40% of patients with eczema have extra-cutaneous atopic co-morbidities including asthma, allergic rhinitis and IgE-mediated food allergies.^{15,24} Other co-morbidities have been suggested from epidemiological studies,²⁵ including depression,²⁶ attention-deficit hyperactivity disorder,²⁷ rheumatoid arthritis and inflammatory bowel disease,²⁸ hypertension and obesity.^{29,30} The shared risk factors and pathomechanisms of this diverse range of co-morbidities are currently unclear.

Importance

Eczema creates a substantial burden of disease with physical, psychological and social impact;¹⁸ suffering is compounded by the multiple atopic³¹ and non-atopic³²

co-morbidities. In the UK, eczema is the most common condition affecting children aged 0 to 9 years³³ with a period prevalence of ~33%³³ and dermatitis is the most prevalent skin disorder in age groups from 20 to >80 years.³³ The total annual UK expenditure on atopic eczema was approximately £465 million in the mid-1990s³⁴ and this cost is projected to rise considerably with the increasing demand for new treatments including biologic agents (described below). On a global scale, dermatitis is the most prevalent skin disease, accounting for an estimated 11 million years lived with disability.³⁵ Eczema accounts for more disability-adjusted life-years than other common inflammatory diseases, including psoriasis, rheumatoid arthritis and inflammatory bowel disease.³⁵ The rising prevalence of eczema, its chronicity and multi-morbidities indicate that primary prevention and developments in treatment are important goals for improving global public health.

Diagnosis

Eczema is diagnosed on the basis of clinical features: ill-defined, erythematous, scaly and pruritic lesions with a clinical history characterised by childhood onset, flexural inflammation, a background of generalised skin dryness (xerosis and/or ichthyosis vulgaris) and associated atopic disease in the patient and/or first degree relatives.¹⁵ Eczema can occur at any body site and non-flexural sites are usually involved in early infancy. However in atopic patients the skin as a whole organ shows impairment in barrier function and a predisposition to inflammation, even in clinically un-inflamed sites. Barrier dysfunction also manifests as skin dryness (in part because of increased water-loss) and susceptibility to viral and bacterial infections.³⁶

Treatment

The main principles of eczema management are allergen and irritant avoidance, emollient use³⁷ and topical or systemic anti-inflammatory treatment.³⁶ Secondary infection with bacteria (most frequently *S aureus*) is commonly associated with eczema exacerbations and should be treated concomitantly. Eczema herpeticum is an infection of herpes simplex virus within areas of skin affected by eczema³⁶ and this requires urgent anti-viral treatment.

The most common allergens leading to sensitisation in childhood are house dust mite, grass pollen, milk, egg and peanut. These allergens induce a type 1, IgE-mediated immediate hypersensitivity reaction rather than eczema itself, but there is evidence that reducing exposure in the subset of sensitised patients can improve eczema control.³⁸ The most common irritants encountered in every-day life are soap and detergents. Clinical experience demonstrates the utility of soap substitutes (emollients) when bathing,³⁷ but a recent clinical trial of emollient bath additives showed they have no significant effect when added to the standard management of paediatric eczema.³⁹ Topical corticosteroids should be applied to areas of active eczema at a potency aimed to induce remission, and a steroid of lower potency or less frequent application used to maintain remission.¹⁵

Second- and third-line treatments include topical calcineurin inhibitors, ultraviolet radiation (principally narrow band UVB phototherapy), oral corticosteroids and systemic immunosuppressants, principally ciclosporin, azathioprine, methotrexate and mycophenolate mofetil. However, only ciclosporin is currently licensed in the UK for adults; systemic treatment for eczema in children is currently unlicensed, but ciclosporin, azathioprine and methotrexate are each used by dermatology specialists in the UK and internationally to treat paediatric eczema cases.^{40,41}

Many new treatments for atopic eczema have emerged in the last 5 years, including small molecules and novel biologic treatments, targeting specific aspects of atopic inflammation.⁴² Dupilumab, the first biologic treatment to be licensed for eczema, is a human monoclonal antibody that binds to the alpha-chain of the interleukin (IL)-4 receptor, inhibiting both the IL-4 and IL-13 pathways.⁴³ It is highly effective in some, but interestingly not all, patients.⁴³⁻⁴⁵ A recent analysis of systematic reviews concluded that there is a paucity of evidence for complementary eczema treatments and non-pharmacological interventions such as specialist clothing; further well-designed clinical trials are needed.⁴⁶

Pathogenesis

Despite the high prevalence of eczema, the pathogenesis of this condition remains incompletely understood. Atopic eczema results from the interplay of skin barrier impairment and immunological dysfunction, both of which are determined by multiple genetic and multiple environmental factors (**Figure 1**).

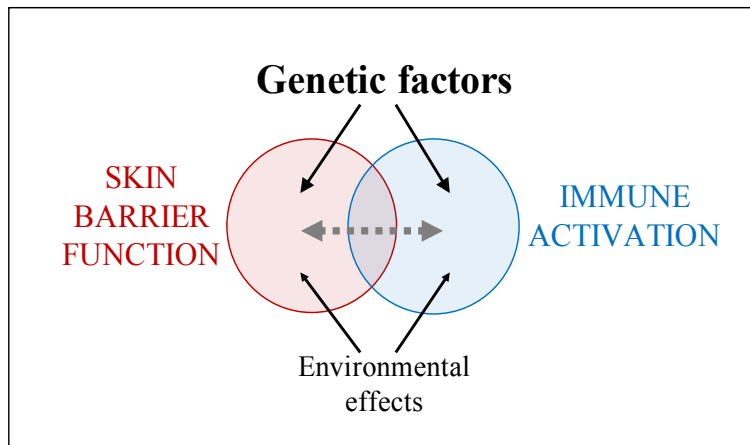


Figure 1. Diagrammatic representation of factors contributing to the pathogenesis of atopic eczema.

Eczema is a complex trait but its high heritability demonstrates the importance of genetic mechanisms in pathogenesis; skin barrier dysfunction and maladaptive immune activation occur as a result of the interactions of multiple genetic and environmental factors; skin barrier and immune activation are themselves closely inter-related.

Atopic eczema is a strongly heritable disease, shown by twin studies and familial clustering. ‘Heritability’ is defined as the proportion of variation within a clinical feature that is attributable to genetic factors.⁴⁷ Twin studies show 72–86 % concordance in monozygotic pairs and 21–23 % in dizygotic twins.^{48,49} A family history of atopic disease is the strongest risk factor for eczema: atopic disease in one parent increases a child’s risk of developing eczema 1.5-fold and the risk is increased ~3-fold if one parent has eczema and ~5-fold if both parents have eczema.^{50,51} The genetic contribution to the development of eczema is 70-80%,^{52,53} which represents high heritability for a complex trait.⁵⁴

This high heritability demonstrates the importance of genetic mechanisms in eczema pathogenesis, but the rapid rise in prevalence over recent decades worldwide indicates the importance of environmental factors.⁵⁵⁻⁵⁹ The notably heterogeneous phenotype of atopic eczema^{18,36} indicates that different aspects of pathogenesis – genetic and environmental, barrier impairment and immune dysfunction – may each play a role to different extents in different cases and at different times in the life-course of disease.

Importance of understanding genetic mechanisms in eczema

Genetic studies have played a major role in transforming the understanding of atopic eczema from a disease seen as an immune-mediated disorder to one that may arise from a primary or secondary skin barrier impairment. Genetic studies have identified a central role for skin barrier impairment in eczema predisposition and perpetuation;²⁴ this brought about a paradigm shift in understanding atopic disease.⁶⁰ The role of Th2-mediated immune dysfunction is also central to atopic inflammation and has proven to be a powerful target for biological therapy in atopic eczema.

Genetic mechanisms controlling skin barrier function remain to be targeted and a more detailed understanding of eczema genetic mechanisms will be required for the era of personalised medicine.⁶¹ Strategic developments in drug discovery will allow the application of genetic insight to the development of novel targeted treatments⁶² and in return a better understanding of genetic mechanisms will facilitate patient stratification in this heterogeneous phenotype. A recent electronic Delphi process defined the ten highest priority research questions for inflammatory skin disease, of which three related specifically to genetic risk mechanisms in eczema.⁶³

Approaches to studying genetic risk in the complex trait of eczema

Atopic eczema is a complex trait (**Figure 1**); strategies for investigating risk have included the study of candidate genes and extreme phenotypes as well as genome-wide analyses, exome and genome sequencing. Mouse models have provided some valuable insights, as reviewed elsewhere,⁶⁴ but murine models are limited in their representation of human skin structure and immune function⁶⁵ and this thesis will focus on human genetic studies.

Candidate gene studies

Many candidate genes have been selected for investigation in relation to eczema pathogenesis because of their theoretical roles in epidermal differentiation, skin or systemic immunity.⁶⁶ Notable findings include a role for variants in the genes encoding IL-4, the IL-4 receptor and IL-13;^{67,68} these associations have been replicated in candidate gene studies and subsequently supported by genome-wide association loci (discussed further, below).

The strongest association and most widely replicated single gene affecting atopic eczema is the gene encoding filaggrin (*FLG*).^{24,69,70} Filaggrin is expressed in the granular layer of the upper epidermis; it is a multifunctional protein contributing to epidermal differentiation and multiple aspects of barrier function.⁷¹ Filaggrin aggregates keratin filaments, contributing to the compaction of corneocytes; filaggrin breakdown releases amino acids that are hygroscopic and acidic and have anti-staphylococcal activity.⁷² Filaggrin deficiency was reported in the common dry skin condition ichthyosis vulgaris (IV) in the mid-1980s⁷³⁻⁷⁵ and IV is known to be a risk factor for atopic eczema. However, because of technical difficulties in sequencing this large and repetitive gene,⁷² loss-of-function mutations in *FLG* were not identified until 2006.⁷⁶ *FLG* null mutations are present in up to 10% of the general population; they cause IV with a semi-dominant mode of inheritance,⁷⁶ such that homozygous (or compound heterozygous) individuals have the full phenotype of dry scaly skin, hyperlinear palms and keratosis pilaris, whilst individuals heterozygous for *FLG* null mutations have a milder dry (xerotic) and/or ichthyotic skin phenotype.⁷⁷ The same null mutations are found in up to 40% of patients with moderate-severe atopic eczema^{24,78} and meta-analysis has shown the risk for eczema in an individual carrying one or more *FLG* loss-of-function mutation(s) to be increased 3-fold (odds ratio 3.12, 95% confidence interval 2.57-3.79).⁷⁰ This illustrates a remarkably strong effect for a single gene in the context of a complex trait. The central effect of filaggrin in skin physiology has been further demonstrated by my work showing a dose-dependent effect of copy number variation within this repetitive gene sequence⁷⁹ and by the effect of atopic inflammatory cytokines which suppress filaggrin expression in the skin resulting in additional barrier impairment.^{80,81}

Study of extreme phenotypes

Atopic eczema varies from a mild, self-limiting skin disease to a severe, chronic disorder affecting the entire skin surface, with multisystem involvement and life-long morbidity. However, severe phenotypes with monogenic inheritance have also offered insight into mechanisms giving rise to atopic skin inflammation. Netherton syndrome (OMIM #256500) is an autosomal recessive condition caused by mutations in *SPINK5*, encoding a serine protease inhibitor, LEKTI. Features of Netherton syndrome include an ichthyotic (dry, scaly) and eczematous skin

phenotype and markedly raised IgE. Several independent studies have shown an association between *SPINK5* variants and atopic eczema.⁸²⁻⁸⁴ This indicates the importance of the protease-antiprotease balance in maintaining an optimal skin barrier function. The control of proteases by protease inhibitors is crucial in the coordinated differentiation and desquamation of the outer epidermis⁸⁵ and also plays an important role in the cutaneous response to allergens, many of which are, or contain proteases.⁸⁶

Another extreme phenotype of atopic skin disease is the syndrome of severe dermatitis, multiple allergies and metabolic wasting (SAM), for which the genes *DSG1* (encoding desmoglein 1) and *DSP* (encoding desmoplakin) have each been implicated.^{87,88} Desmosomes are intercellular junctions which provide mechanical strength to the skin and are degraded in a controlled way during physiological desquamation;⁸⁵ they also play a key role in cell signaling.⁸⁹ The finding of mutations in *DSG1* and *DSP* causing SAM indicates the importance of desmosomal proteins in protecting against local and systemic atopic inflammation. Other ‘human knock-out’ models include *CARD11* mutations (causing systemic atopic inflammation), *DSG1* and *DSP* mutations (causing severe dermatitis, multiple allergies and metabolic wasting) and various immunodeficiency syndromes with atopic eczema-like skin inflammation, such as Wiskott-Aldrich, caused by mutations in *WAS*.⁶⁵

Importantly, there are forms of more extreme skin barrier impairment which do not appear to predispose to atopic inflammation, such as epidermolysis bullosa. Furthermore, recent molecular profiling of patients with several different rare monogenic ichthyoses has suggested they may share the feature of Th17 immune activation.⁹⁰ This illustrates the complex and specific role of transcutaneous sensitization in allergic disease.

Genome-wide analyses

A genome-wide association study (GWAS) aims to identify regions of the genome that are associated with a specific trait or disease. The technique compares the frequency of single nucleotide polymorphisms (SNPs) between cases and controls, similar to a massive case-control study. Large numbers (one-two million) of SNPs are assessed, sampling regions across the whole genome and large numbers

(thousands - tens of thousands) of cases and controls are needed to achieve sufficient statistical power. Results are summarized in the form of a Manhattan plot. GWAS is most effective in defining genetic risk loci for common complex traits, because a sufficient sample size can be obtained. It is important to note that the effect sizes detected by GWAS can range from odds ratio (OR) >2 (*ie* risk more than doubled) to very small effect sizes (OR 1.1 or lower *ie* $<10\%$ increased risk) because complex traits are a result of multiple genetic effects.

The first genome-wide association study (GWAS) for atopic eczema was published in 2009, representing the white European population.⁹¹ This identified the locus on chromosome 1q21.3 (the epidermal differentiation complex which includes *FLG*) as well as a locus on chromosome 11q13.5, in an intergenic region of unknown function; subsequent GWAS analyses have replicated these findings. A meta-analysis combining published and previously unpublished data was completed in 2015⁹² which included over 15 million genetic variants in $>20,000$ cases and $>95,000$ controls from populations of European, African, Japanese and Latino ancestry, followed by replication in $>250,000$ eczema cases and controls. This powerful meta-analysis detected 10 additional risk loci, bringing the total number of eczema risk loci identified to date to 31. The majority of GWAS loci are in intergenic or intronic regions for which the function remains unclear, but where functions can be inferred these have largely been attributed to skin barrier development, innate and acquired immunity. The most recently identified loci have been proposed to identify genes with roles in the regulation of innate host defence and T cell function, supporting a possible role for autoimmune mechanisms in eczema pathogenesis.^{92,93}

Whole exome sequencing (WES) has contributed to the diagnosis of extreme phenotypes in rare diseases (including SAM, described above⁸⁸) and has offered greater detail in the search for rare variants causing common diseases. To date only one small WES study has been reported for eczema, in which 22 Ethiopian patients with ichthyosis vulgaris and atopic eczema were sequenced. This identified possible disease-causing variants in *FLG* and related genes as well as some novel candidate genes in a heterogeneous pattern of risk.⁹⁴ Exome sequencing data has been reported for *FLG* and related genes⁹⁵ as well as for the HLA region,⁹⁶ but larger studies will be required to systemically identify rare variants contributing to atopic eczema.

Further, whole genome sequencing and painstaking functional studies will be required to fully assess the complex contribution of non-coding genetic variants, which is anticipated to be substantial.^{97,98}

Transcriptome analyses

The accessibility of skin tissue has allowed the detailed comparison of gene expression patterns in lesional (active eczematous skin) compared with non-lesional (uninflamed skin on a patient with eczema) and normal skin (from a non-atopic individual). Whole transcriptome analyses, using RNA sequencing techniques provide more comprehensive assessment and more detailed quantification of mRNA than previous microarray studies. Several studies have identified networks of differentially expressed genes involved in keratinocyte differentiation in the epidermis, innate and acquired immune responses as well as lipid metabolism.⁹⁹⁻¹⁰¹ Laser capture microdissection has recently been used to separate the dermal and epidermal gene expression signatures, identifying some novel immune and barrier genes¹⁰² which require further validation.

Single cell analysis

Most of the molecular analyses on skin to date have been carried out using whole skin biopsies, or epidermal samples. However single cell analysis is now feasible for DNA and RNA sequencing, as well as protein analysis.¹⁰³ These techniques offer the prospect to study individual cells, define new cell types and gain insight into the functional and structural heterogeneity of skin as a complex organ. The Human Cell Atlas is an international collaboration to make single cell analytical data available to researchers¹⁰⁴ and the skin component of this atlas is eagerly awaited. Several research laboratories have already released data and tools to allow the interrogation of skin transcriptome analysis, for example murine data from the Kasper lab.¹⁰⁵

***In vitro* modelling of genetic effects**

Knockdown of candidate genes using RNA inhibition (RNAi)

Small interfering RNA (siRNA) can be used to bring about a selective reduction in gene expression based on complimentary base pairing. siRNA is a double-stranded molecule 20-25 nucleotides in length. When transfected into the cell type of interest

(principally primary human keratinocytes for eczema studies) siRNA binds with a complex of proteins to form an RNA-induced silencing complex (RISC) in which the double-stranded siRNA disassociates to become single-stranded. The single stranded siRNA can then bind to complementary mRNA, bringing an Argonaut protein into proximity to catalyse the cleavage of this mRNA. Translation is prevented, hence silencing the gene expression.

siRNA-mediated knockdown can be carried out in primary cells of low passage, using chemically-enhanced transfection, to minimise interference with physiological conditions. There is, however, still an innate immune response to the siRNA and scrambled or non-targeting siRNA is an essential control condition. The duration of knockdown is short-lived (hours-days, depending on the rate of mRNA turnover) and this can be a limitation to the technique.

Short hairpin RNA (shRNA) is another method for RNAi. shRNA is a synthetic double-stranded RNA molecule with a tight hairpin turn; it is synthesised in the cell type of interest using a plasmid introduced by viral or bacterial vector transfection. The vector integrates into host DNA and shRNA is transcribed in the nucleus, processed by dicer and loaded into the RISC. The sense strand is degraded and the antisense (guide) strand directs RISC to mRNA that has a complementary sequence where RISC cleaves or represses translation of the mRNA. An advantage of shRNA knockdown is the longer lasting duration of effect compared to siRNA; a disadvantage is the need for vector transfection and integration, producing a less physiological condition in primary cells or cell lines.

CRISPR-cas9 gene editing

CRISPR (clustered regularly interspaced palindromic repeat) sequences are found in bacterial DNA and form part of their immune response to phage infection. Cas9 (CRISPR-associated protein 9) is an enzyme that cleaves DNA selectively at sequences containing the CRISPR motif. In 2012 it was reported that this mechanism can be exploited for genetic engineering;^{106,107} guide-RNAs are used to direct the cas9 enzyme to cleave DNA in precisely-targeted editing. Application of CRISPR-cas9 allows the effects of genetic variation to be tested directly and the technique has revolutionised molecular biology. This cost-effective and relatively easy-to-use technology has allowed researchers to precisely and efficiently target, edit, modify

and mark genomic loci in a wide range of cells and organisms.¹⁰⁸ Within dermatology, CRISPR-cas9 can be used to investigate candidate genes *in vitro*, allowing the study of their effect on replicated samples with a controlled genetic background.

Gene over-expression

Over-expression of candidate genes is a key technique to assess their effect. Stable over-expression of a gene of interest can be achieved using plasmid transfection, lentivirus transduction or CRISPR-cas9 editing.

In vitro models

Keratinocyte culture *in vitro* was first established by Rheinwald and Green in 1975,¹⁰⁹ opening up the opportunities for detailed study of this cell type. Multi-layered keratinocyte cultures recapitulate many features of human epidermis including morphology, spatiotemporal expression of terminal differentiation markers and lipid content.^{110,111} Multiple different two-dimensional (keratinocyte culture) and three-dimensional (organoid) models of skin and eczema have been described, as recently reviewed.⁶⁵ Some organoid models are epidermal-only whereas others include a model dermis as well as epidermis. The model dermis includes fibroblasts; these cells may be suspended in a fibrin or collagen gel, or an artificial matrix; alternatively, a de-epidermalised dermis may be used. Co-culture with T cells and neurons has been piloted but these models are not yet well validated.⁶⁵

Explanted whole skin has also been used for *ex vivo* experimentation. Explanted skin has the advantage of including intact skin adnexae and a complement of resident cell types, but it is tissue that is inevitably beginning a process of demise.

Two main approaches can be used to model eczema pathophysiology *in vitro*: culturing normal human keratinocytes with eczema-relevant cytokines (IL-13 and IL-4);¹¹² alternatively gene silencing or *FLG* mutant keratinocytes can be used to model a filaggrin-deficient state.¹¹³ Organoid models deficient in filaggrin show a lack of keratohyalin granules, increased paracellular permeability^{114,115} and proteomic features consistent with eczema.¹¹⁶ The use of normal keratinocytes (as opposed to cells extracted from eczema biopsies) allows the investigation of specific,

selected genetic mechanisms without the complex and confounding effects of multiple genetic and epigenetic risk variants that are co-inherited in atopic eczema.

Organoid models allow the investigation of tissue-specific genetic effects and the opportunity for testing specific eczema candidate genes, by knockdown, over-expression, or CRISPR-cas9 editing of genes of interest. Three-dimensional organoid models of human skin (epidermis and dermis, **Figure 2**) offer a unique opportunity to investigate genetic effects in a tissue context.

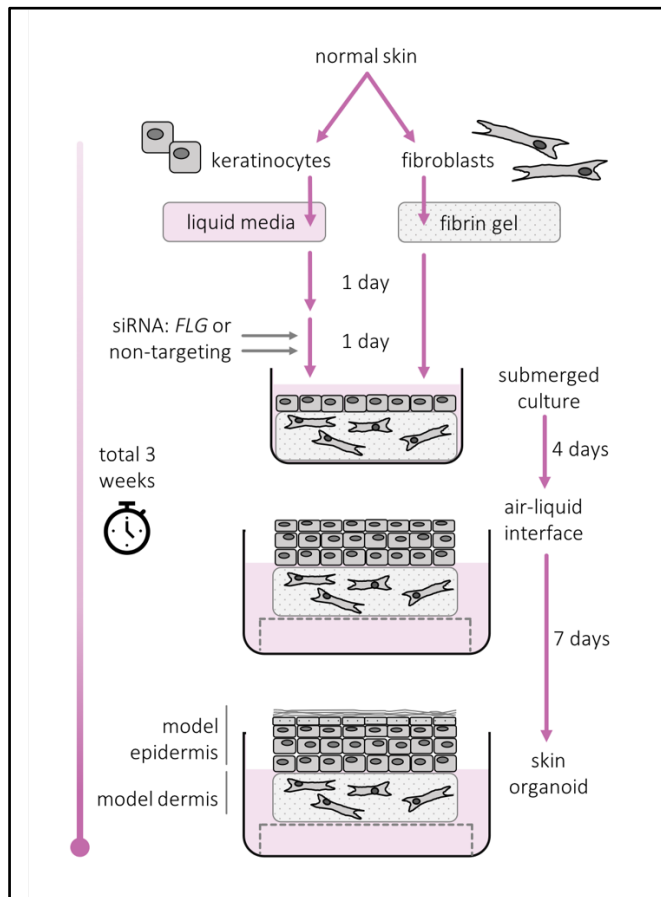


Figure 2. Workflow showing methods used to produce skin organoid model for candidate gene analysis *in vitro* in the Brown laboratory.

This human skin organoid takes a total of 3 weeks to produce, but it recapitulates gene expression patterns, biochemical and functional features of epidermis *in vivo*. In this model candidate genes are knocked down using siRNA transfection of primary keratinocytes and the effect may be observed in mature organoids 10 days later. This figure has been reproduced from Elias *et al.* 2019.¹¹⁷

Functional analyses in vitro

Organoid skin grown at the air-liquid interface develops a competent epidermal barrier with biophysical properties analogous to human skin. This allows the quantitative measurement of functional parameters, including hydrophilic dye penetration, water content in the stratum corneum (measured by transepidermal electrical resistance, or capacitance) and water evaporation from the surface (equivalent to transepidermal water loss, TEWL, *in vivo*).^{65,114,118,119}

CHAPTER TWO: Genetic risk in atopic eczema

***FLG* null mutation creates a strong genetic risk for atopic eczema**

Filaggrin haploinsufficiency affects risk of mild, moderate and severe eczema

The identification of genetically-determined skin barrier dysfunction in eczema pathogenesis 10 years ago⁶⁹ brought about a paradigm shift in understanding atopic disease. The strong and highly significant association of *FLG* null mutations with atopic eczema has subsequently been replicated in numerous independent studies, including case-control studies, family studies and unselected population cohorts. Two independent meta-analyses carried out in 2009 estimated the odds ratio of developing atopic eczema in association with *FLG* null genotype to be 4.78¹²⁰ and 3.12⁷⁰ *i.e.* individuals with one or more *FLG* loss-of-function mutations have a 3-5 times greater risk of developing atopic eczema.

The eczema sub-phenotypes that are most strongly associated with *FLG* null mutations are early-onset, severe, and persistent disease^{121,122} and eczema with the associated ‘extrinsic’ features of raised total IgE and allergic sensitization.^{123,124} These patients are most frequently referred to secondary care. However, population cohort studies include atopic eczema of milder severity and this phenotype has also demonstrated significant association with *FLG* null mutations.^{19,31,125} Mild-to-moderate eczema shows a lower odds ratio than severe disease and its association with *FLG* null mutations may be significant only in individuals carrying two null mutations.¹⁹

Filaggrin deficiency also increases risk of irritant and allergic contact dermatitis

Following on from the strong association of atopic eczema with *FLG* null mutations, several other subgroups of eczema were investigated for their link with filaggrin. There is a well-recognized genetic susceptibility to contact dermatitis,¹²⁶ but it is often difficult to distinguish the subgroups of irritant and allergic contact dermatitis from atopic eczema; hence, these genetic studies are difficult to interpret.¹²⁷⁻¹²⁹ Studies on *FLG* genotype as a risk factor for experimentally-induced irritant dermatitis have given somewhat conflicting results,^{130,131} and this may be because of methodological differences in the concentration of irritant. Subsequent studies reported no association of *FLG* genotype with allergic contact dermatitis defined by

a positive patch test result to one or more substance,¹³² nor with a subset of patients having both atopic eczema and contact allergy.¹³³ In contrast, *FLG* null genotype has shown significant association with nickel sensitization, but only in a subgroup of cases reporting intolerance to costume jewellery¹³⁴ and in a subgroup of women who had not had their ears pierced.¹³⁵ This illustrates the importance of environmental exposures in allergy development, as well as the need for careful phenotype definition in genetic studies.

FLG null mutations contribute to risk of multiple atopic disorders

The term ‘atopic march’¹³⁶⁻¹³⁸ refers to the observation that atopic eczema may precede the development of food allergies, asthma, and allergic rhinitis in a temporal sequence, although it is now recognised that this sequence and trajectory are far from inevitable.^{137,139-141} *FLG* null mutations represent a risk factor for each step in the atopic march: atopic eczema;⁷⁸ allergic sensitization;¹²⁰ the subgroup of asthma in association with eczema;^{19,31,142} allergic rhinitis;^{142,143} and IgE-mediated peanut allergy.¹⁴⁴ *FLG* null alleles are therefore a significant risk factor for all aspects of atopy, with differing odds ratios for each specific phenotype. The odds ratio (OR) for eczema is ~1.3, whilst allergic rhinitis is ~2.8, atopic asthma ~1.5 and peanut allergy ~3.8. These studies have brought new understanding about the need to address skin barrier dysfunction as a driver of allergic disease.

Pathomechanisms associated with filaggrin-deficiency are identified by in vitro studies

As described above, filaggrin and its precursor profilaggrin are multifunctional proteins and it is unclear which of these functions are most relevant to eczema pathogenesis. The precise molecular pathways leading from filaggrin haploinsufficiency to atopic diathesis have been the focus of a large amount of research work over recent years. The accessibility of human skin and established methods for the culture of primary cells and immortalised cell-lines, in addition to organoid modelling has facilitated progress considerably.⁶⁵

One promising line of enquiry is the role of aryl hydrocarbon receptor (AhR) signalling in increasing filaggrin expression^{145,146} but the control of keratinocyte differentiation is complex and some aspects of the AhR signalling pathway are

detrimental to skin health.¹⁴⁷ The cytokines most prominent in atopic inflammation, IL-4 and IL-13, have been shown to reduce *FLG* expression.¹⁴⁸ TNF- α ⁸¹ and IL-17¹⁴⁹ may also downregulate filaggrin expression. Caspase-14 is required for filaggrin breakdown, to release amino acids as part of the natural moisturising factors in the stratum corneum¹⁵⁰ and an mTORC1/AKT1/Cathepsin H axis has been described, playing a role in the control of filaggrin expression and processing in skin.¹⁵¹ There is evidence that hypoxia-inducible factors regulate filaggrin expression¹⁵² and filaggrin-deficiency itself promotes an inflammatory phenotype.¹¹⁶ It is, however, salutary to note that multiple gaps in knowledge persist, including questions about the control of filaggrin expression, the relative role of filaggrin and its breakdown products in structural versus microbiological and immunological barrier functions. Despite the reported opportunities for therapeutic targeting, no treatments are currently available that can directly upregulate filaggrin expression for the treatment or prevention of eczema.

***FLG* null mutations predispose to some, but not all, forms of inflammatory disease**

FLG mutations in other inflammatory conditions

The strong effect of filaggrin on atopic inflammation does not extend to all inflammatory disease. Psoriasis shows association with the 1q21 locus in genome-wide studies¹⁵³ and this locus is within the epidermal differentiation complex (EDC), a dense cluster of over 60 genes, including *FLG*.¹⁵⁴ Filaggrin shows increased expression in psoriasis, but *FLG* mutations (loss- or gain-of-function) are not associated with psoriasis in the multiple European populations studied to date.¹⁵⁵⁻¹⁶⁰ Variants in *FLG* have been associated with psoriasis in Chinese populations^{161,162} but the functional effect of these variants has not been defined, therefore an association with other genetic effects in linkage disequilibrium remains a possibility. The psoriasis-chromosome 1q21.3 association appears more likely to be driven by a deletion affecting two of the late cornified envelope genes (*LCE3C* to *LCE3B*) within the EDC.^{163,164}

In one report *FLG* null mutations showed a possible protective effect against acne vulgaris¹⁶⁵ but a larger and more adequately powered study in the Singaporean Chinese population effectively excluded an association.¹⁶⁶ Other inflammatory

barrier diseases, including Crohn's disease, ulcerative colitis, and sarcoidosis, share common susceptibility loci with atopic eczema¹⁶⁷ but do not show association with *FLG* null mutations.^{168,169} This indicates that additional genetic mechanisms are responsible for barrier perturbations in these diseases.

Filaggrin haploinsufficiency in autoimmune skin disease

Autoantibody formation to citrullinated profilaggrin is a sensitive and specific serological marker for rheumatoid arthritis¹⁷⁰ and can predate the onset of joint disease.¹⁷¹ Levels of anti-filaggrin antibody may also correlate with arthritis activity.¹⁷² However, filaggrin is not expressed in articular tissues and these autoantibodies appear to be produced as a result of cross-reaction between deiminated peptide sequences, which are present within fibrin chains in the synovium and deiminated sequences produced during the post-translational processing of profilaggrin. Anti-filaggrin antibodies are therefore not believed to be pathogenic.¹⁷³ *FLG* null mutations R501X, 2282del4, and 3702delG have been studied in rheumatoid arthritis patients and controls,¹⁷⁴ and *FLG* mutation carriers showed no increased risk of developing rheumatoid arthritis. However, in a separate study, *FLG* heterozygotes did show significantly elevated levels of autoantibodies to citrullinated peptides compared with controls¹⁷³ and *FLG* null mutations may therefore contribute to the development of humoral autoimmunity in early rheumatoid arthritis¹⁷⁴ by an as yet undefined mechanism. Alopecia areata is a tissue-specific autoimmune disease and genetic factors make a significant contribution to its aetiology.¹⁷⁵ Alopecia areata is known to be associated with atopic disease and it has been shown that comorbidity with atopic eczema, as well as prevalent *FLG* null variants (R501X and 2282del4), predict a more severe form of alopecia areata.^{176,177} The specific mechanism remains unknown.

FLG null mutations and chronic photosensitivity

Another inflammatory condition associated with atopic eczema is chronic actinic dermatitis (CAD). CAD is a photosensitivity dermatitis in which individuals show abnormal reactions to UVB, UVA and in severe cases to visible light, plus an increased incidence of contact sensitivity to airborne allergens. Whilst *FLG* mutations may obviously contribute to the underlying atopic eczema, they are not strongly associated with CAD.¹⁷⁸

Taken together, these observations indicate that filaggrin deficiency creates a pathogenic tissue environment in which atopic inflammation predominates, but the propensity to other forms of non-atopic inflammation is also of importance.

Genome-wide association studies have identified eczema risk loci giving mechanistic insight

GWAS loci confirm and extend knowledge

GWAS have been performed in large populations sampled from European and Asian ethnicities. Some eczema risk loci have confirmed pre-existing knowledge, including the role of skin barrier and type 2 inflammation in the pathogenesis of this disease: the epidermal differentiation complex on chromosome 1q21.3 includes *FLG* and the cytokine cluster on chr5q31.1 includes genes encoding IL-13 and IL-4. Other loci have provided newer insights, including evidence for auto-immunity⁹² and a role for Langerhans cells, indicated by variants in a locus on 2p13.3 which affect the expression levels of CD207/langerin in skin.⁹²

An extension to GWAS, focusing on protein-coding variants, used exome genotype and skin transcriptome data.¹⁷⁹ This study identified an additional 12% of AD heritability explained by rare protein-coding variation in genes including *IL4R*, *IL13*, *JAK1*, *JAK2* and *TYK2* (current therapeutic targets in the IL-13 pathway) and novel candidate genes *DOK2* and *CD200R1* for which eczema susceptibility mechanisms remain to be defined.¹⁷⁹

GWAS of multiple atopic traits has shown considerable overlap in genetic risk profiles for eczema, asthma and allergic rhinitis^{180,181} attributed predominantly to lymphocyte-mediated immunity. It is interesting to note that only two loci indicate eczema-specific effects and these are both within the epidermal differentiation complex on chromosome 1q21.3, attributed to *FLG* and *HRNR-RPTN*.¹⁸⁰

The GWAS performed to date have been conducted in white European and selected Asian populations. The lack of ethnic diversity in genetic research has been highlighted as a critical weakness in the field, in terms of equity in access to medical and scientific knowledge and also as a missed opportunity for genetic discovery.¹⁸² Eczema GWAS studies in more diverse ethnicities are ongoing.

Epistatic effects are likely to be common but most remain undefined

Genetic risk loci are likely to have multiple interactions in their contributions to complex traits,¹⁸³ but the identification of these interactions using a statistical approach is challenging, because of the burden of multiple testing. One epistatic interaction that appears to occur is the effect of *FLG* null mutations in the context of a variant on chromosome 11q13.5: the effects of these two GWAS peaks are multiplicative at a population level.¹⁸⁴ This indicates that an interaction or overlap in the molecular pathogenesis is likely. Another epistatic effect has been demonstrated in variants of the high affinity IgE receptor gene *FCER1A*¹⁸⁵ indicating that multiple variants may have synergistic effects on this pathomechanism.

Relevance of genetic risk mechanisms for clinical practice*FLG null mutations produce a recognisable clinical phenotype*

Ichthyosis vulgaris in an individual with two *FLG* null mutations (*i.e.* homozygous or compound heterozygous) is a triad of ichthyosis with fine scaling, palmar hyperlinearity and keratosis pilaris. These features indicate *FLG* null genotype without the need for genetic testing.⁷⁷ Furthermore, when combining all the milder clinical signs that may be attributable to *FLG* haploinsufficiency (atopic eczema, ichthyosis, xerosis, palmar hyperlinearity, and keratosis pilaris), *FLG* null mutations are seen to be highly penetrant.⁷⁷ Careful clinical examination of patients and their relatives can therefore be used to implement understanding of *FLG*-related genetic risk in the clinical setting. However, there are likely to be multiple other genetic effects of relevance to clinical practice which we cannot yet apply in clinic.

Skin barrier impairment is a central pathomechanism

The filaggrin story demonstrates how the study of a monogenic disorder can provide insight into complex trait disease, and the striking significance of *FLG* null mutations as a genetic risk factor for atopic eczema has placed a new focus on the role of barrier impairment in eczema pathogenesis. This offers the opportunity for therapeutic intervention – simple emollients to protect and improve skin barrier function – and also a rationale for patient education, encouraging the use of emollients, through an explanation of skin barrier impairment.

In light of the strong association between atopic eczema and peanut allergy, it was important to consider whether the association of *FLG* genotype with peanut allergy occurs through atopic eczema on the skin,¹⁸⁶ or whether the risk occurs as a genetic mechanism independent of eczema, in order to target prevention appropriately. It is likely that both mechanisms are active, but there are various sources of evidence favouring an effect of *FLG* null genotype that is independent of eczema. In a study that I co-led¹⁴⁴ the association of *FLG* genotype with peanut allergy was not dependent on co-existent eczema. A separate study showed a dose-dependent effect of environmental peanut exposure on children with *FLG* null mutations, after adjustment for infantile eczema, supporting the hypothesis that peanut allergy can develop through transcutaneous sensitization even without eczema.¹⁸⁷ Therefore it appears that risk of peanut allergy is mediated via the *FLG* genetic risk independently, or at least it is not dependent on, the presence of eczema.

Stratification of patients in clinical trials

Given the strong and central effect of *FLG* genotype on eczema, this has been used to stratify clinical trials, to assess whether patients with filaggrin haploinsufficiency may be more or less likely to benefit from a specific treatment. There is some evidence that *FLG* wild-type genotype is associated with a more favourable response to omalizumab therapy for atopic eczema.¹⁸⁸ In contrast there was no difference in response to silk clothing for the treatment of eczema in a carefully designed randomised controlled trial.^{189,190}

Opportunities for therapy development

Many of the novel biologics and small molecule drugs for treating atopic eczema have been designed to target specific aspects of the atopic immune response.⁴² These pathways were previously identified by research in the field of immunology, but their importance has been supported by GWAS hits.

Novel therapies targeting aspects of skin barrier formation or function are yet to show a transformative effect on eczema management. Conventional simple emollient formulations transiently improve water retention properties of skin *in vivo*.¹⁹¹ Novel emollients aiming to replace physiological doses of ceramides and amino acids to the stratum corneum have been developed. These complex preparations show a more long-lasting increase in hydration parameters in paediatric eczema¹⁹² and elderly

xerotic skin.¹⁹³ The position for these more expensive emollients in atopic eczema management remains to be defined.

An attractive opportunity arising from the application of knowledge of genetic risk mechanisms is the possibility of primary prevention of eczema and other atopic diseases, which would have an enormous public health benefit. This will be considered further in the critical appraisal in Chapter Four.

CHAPTER THREE: Body of work to be considered for PhD

Selected publications from a five-year period, 2014 to 2019

The publications that I wish to be considered within this thesis are as follows:

- [1] Cole C, Kroboth K, Schurch NJ, Sandilands A, Sherstnev A, et al., Watson RM, McLean WHI, Barton GJ, Irvine AD and **Brown SJ**. Filaggrin-stratified transcriptome analysis of paediatric skin identifies mechanistic pathways in atopic dermatitis. *J Allergy Clin Immunol*. 2014; 134(1):82-91.

- [2] Forbes D, Johnston L, Gardner J, MacCallum SF, Campbell LE, Dinkova-Kostova A, McLean WHI, Ibbotson SH, Dawe RS and **Brown SJ**. Filaggrin genotype does not determine the skin's threshold to UV-induced erythema. *J Allergy Clin Immunol* 2016;137(4):1280-2.

- [3] Elias MS, Wright SC, Nicholson WV, Morrison KD, Prescott AR, Ten Have S, Whitfield PD, Lamond AI and **Brown SJ**. Functional and proteomic analysis of a full thickness filaggrin-deficient skin organoid model. *Wellcome Open Res*. 2019, 4:134.

- [4] Elias MS, Wright SC, Remenyi J, Abbott JC, Bray SE, Cole C, Edwards S, Gierlinski M, Glok M, McGrath JA, Nicholson WV, Paternoster L, Prescott AR, Have ST, Whitfield PD, Lamond AI and **Brown SJ**. EMSY expression affects multiple components of skin barrier with relevance to atopic dermatitis. *J Allergy Clin Immunol*. 2019 Aug;144(2):470-481.

- [5] Paternoster, L Savenije OEM, Heron J, Evans DM, Vonk JM, Brunekreef B, Wijga AH, Henderson AJ, Koppelman GH* and **Brown SJ***. Identification of atopic dermatitis subgroups in children from two longitudinal birth cohorts. *J Allergy Clin Immunol* 2018;141(3):964-971. [* denotes equal contribution]

- [6] Baurecht H,* Hotze M,* Brand S, Büning C, Cormican P, Corvin A, Ellinghaus D, Ellinghaus E, Esparza-Gordillo J, Fölster-Holst R, Franke A, Gieger C,

Hubner N, Illig T, Irvine AD, Kabesch M, Lee Y AE, Lieb W, Marenholz I, McLean WHI, Morris DW, Mrowietz U, Nair R, Nöthen MM, Novak N, O'Regan GM, PAGE consortium, Schreiber S, Smith C, Strauch K, Stuart PE, Trembath R, Tsoi LC, Weichenthal M, Barker J, Elder JT, Weidinger S,* Cordell HJ* and **Brown SJ**.* Genome-wide comparative analysis of atopic dermatitis and psoriasis gives insight into opposing genetic mechanisms. *Am J Hum Genet*. 2015; 96(1):104-20. [* denotes equal contribution]

The contributions made by each of these publications to the understanding of atopic eczema are summarised in **Figure 3**. The rationale, key aspects of study design and main findings for each publication will be considered in turn.

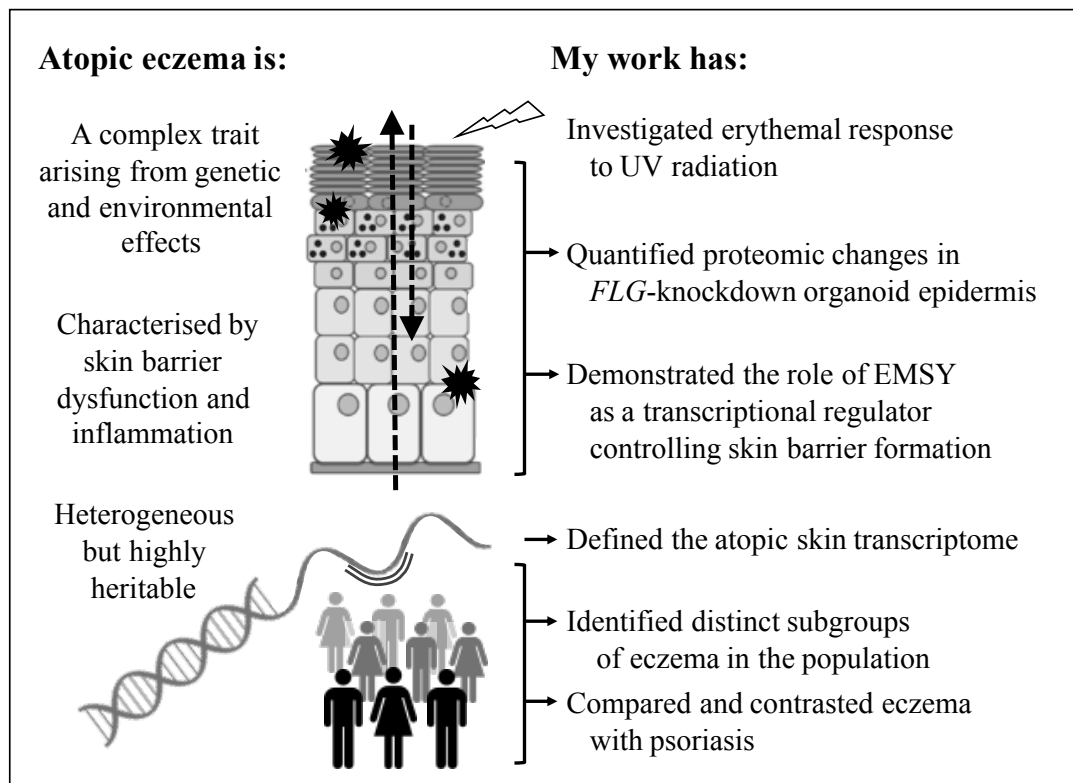


Figure 3. Diagrammatic summary of key features of atopic eczema pathogenesis and my contributions to understanding its pathogenesis.

The diagram of epidermal structure is modified from a diagram created by M. Elias; the contributions from each of my publications selected for this PhD thesis^{101,117,194-197} are listed in the right half of the figure.

[1] Filaggrin genotype has a global effect on skin transcriptome, with relevance to atopic eczema

Single molecule direct RNA sequencing of skin samples

Having previously described the clinical phenotype of filaggrin haploinsufficiency in detail as part of a population-based study,⁷⁷ I proceeded to study this at a molecular level. I chose to use a forefront technique at the time, single molecule RNA sequencing, because this allows analysis of a small quantity of RNA (such as that available from paediatric skin biopsies). The technology captures and sequences mRNA from the poly-A tail. Digital quantitative analysis is facilitated by counting each messenger RNA (mRNA) molecule.¹⁹⁸

The skin transcriptome varies considerably in different body sites, in different sexes and at different ages, as demonstrated on a macroscopic scale by the clinical features and on a biochemical and molecular genetic level.^{199,200} I therefore recruited volunteers specifically for control sampling, to allow the closest possible match in age, sex and body site. The 26 eczema cases were aged between 6 and 16 years; controls were aged 16-19 years.

Stratification by FLG genotype

Transcriptome analysis creates a large and complex dataset and this is further complicated by the inter-individual variation that is inherent in clinical samples. Since *FLG* genotype has the single greatest genetic effect on eczema phenotype,^{18,24,70,72,77,201} this was used to stratify the transcriptome data. In this study, because of the relatively small numbers of cases and controls, it was possible to carry out very comprehensive *FLG* genotype analysis, including next generation sequencing of all coding regions in *FLG* and confirmation of mutations by Sanger sequencing.

Main findings

2430 genes were differentially expressed (false discovery rate (FDR) $P < 0.05$) of which 211 genes were upregulated and 490 downregulated >2 -fold. The gene ontology terms 'extracellular space' and 'defence response' were enriched, whereas 'lipid metabolic processes' were downregulated. The subset of *FLG* wild-type cases showed dysregulation of genes involved with lipid metabolism, whereas filaggrin

haploinsufficiency affected global gene expression and was characterized by a type 1 interferon-mediated stress response.

[2] Filaggrin haploinsufficiency does not affect the skin's threshold to UV-induced erythema

Evidence for filaggrin breakdown products contributing to skin photoprotection in vitro requires testing in vivo

A role for filaggrin breakdown products in photoprotection is suggested on the basis of their biochemical and physicochemical properties.⁷² Filaggrin is a histidine-rich protein⁷⁵ and histidine is metabolized to trans-urocanic acid (trans-UCA). The photoisomerization of trans-UCA to cis-UCA²⁰² produces a molecule with an action spectrum of 280–310nm,²⁰³ which is within the UVB range. There is evidence from *in vitro* studies that cis-UCA has immunomodulatory actions in human keratinocytes and leukocytes.^{203,204} There is also *in vitro* evidence from an organotypic siRNA *FLG* knockdown model that lack of filaggrin leads to a reduction in UCA concentration and an increased sensitivity to UV-induced apoptosis.¹¹⁴ Histidinemia mice, deficient in cutaneous UCA because of a mutation in *Hal*, the gene encoding histidase, show increased propensity to UVB-induced DNA damage.²⁰⁵ However, evidence of a photoprotective effect in humans *in vivo* was lacking.

Detailed phototesting of healthy volunteers

In collaboration with expert photobiologists (Drs Robert Dawe and Sally Ibbotson) in Dundee, I gained access to detailed monochromator phototesting data that had previously been generated for 71 adult volunteers with clinically normal skin, as part of a drug phototoxicity study. All participants were of white European ethnicity; this was required to minimise inter-individual variation in photo-response and to standardise *FLG* genotyping as far as possible. All of the participants were requested to provide saliva samples to allow testing for the 6 most prevalent loss-of-function mutations in *FLG* in this white European population (R501X, 2282del4, R2447X, S3247X, 3673delC, and 3702delG) using previously published methodology.^{19,206}

Main findings

Contrary to expectations, there was no significant effect of *FLG* genotype on UV-induced skin erythema. This study was sufficiently powered to exclude a large effect of *FLG* genotype on photosensitivity (>1.8-fold difference in MED) at any of the wavebands tested. Furthermore, monochromator phototesting did not indicate a differential erythematous sensitivity within the wavelengths representing UVB, as would be predicted from the known absorption spectrum of UCA.

[3] The filaggrin-deficient skin organoid is a valuable model

Opportunities to investigate genetic mechanisms in skin

Skin is more amenable than the many other human organs to modelling and genetic manipulation *in vitro*; organoid cultures facilitate a bridge in understanding from genetic and cellular level to physiological effects and potential therapeutic targets. A skin organoid created from adult stem cells²⁰⁷ (primary fibroblasts and basal keratinocytes) forms stratified layers, recapitulating the structure and gene expression patterns of human skin.^{117,195,208} This avoids some of the limitations of mouse models that are most pertinent to inflammatory skin disease, including the limited numbers of cell layers in the epidermis, frequent hair follicles and variation in genetic effects on different background strains.⁶⁵

Optimisation of model for assessment of molecular and functional parameters in skin barrier

Primary human keratinocytes and donor-matched primary fibroblasts from healthy individuals were used to create skin organoid models with and without siRNA-mediated knockdown of *FLG*. Biological and technical replicate sets of organoids were assessed using histological, functional and biochemical measurements.

Quantitative global mass spectrometry proteomics was used to identify >8000 proteins per sample. The model was optimised to produce sensitive, reproducible functional and biochemical measurements.¹¹⁷

Main findings

FLG knockdown led to subtle changes in histology and ultrastructure. Immature organoids showed evidence of barrier impairment with *FLG* knockdown, but the

mature organoids showed no difference in transepidermal water loss, water content or dye penetration. Gene ontology (GO) and pathway analyses identified an increase in transcriptional and translational activity but a reduction in proteins contributing to terminal differentiation, including caspase 14, dermokine, AKT1 and TGF-beta-1. The GO term ‘axon guidance’ and aspects of innate and adaptive immunity were represented in both the up-regulated and down-regulated protein groups. This work provides further evidence for keratinocyte-specific mechanisms contributing to immune and neurological, as well as structural, aspects of skin barrier dysfunction. All methods and data were shared in detail, as a resource for the field.

[4] An EMSY-deficient organoid model reveals effects on multiple components of skin barrier formation and function

EMSY is the nearest gene to the risk locus on chr11q13.5 and it has no known function in skin

The chr11q13.5 locus, identified in multiple GWAS, lies in a large (~104kb) intergenic region between *EMSY* and *LRRC32* in one topologically-associated domain. This locus has also been associated with other immune-mediated barrier disorders including atopic sensitisation^{180,209} and inflammatory bowel disease.²¹⁰ *EMSY* encodes a BRCA2-interacting protein, implicated in nuclear hormone regulated transcription and repression²¹¹ but it has not previously been studied in skin. The organoid model, optimised for barrier assessment, is therefore an ideal platform with which to assess a role for this candidate gene in atopic eczema.

EMSY knockdown and over-expression, with functional and biochemical analyses

The commercially available antibodies for EMSY required time-consuming testing and careful validation. Knockdown of *EMSY* in primary keratinocytes was achieved using siRNA; importantly, persistence of knockdown was demonstrated at day 10 in organoid culture. mRNA showed a mean 36% reduction and EMSY protein was reduced to ~50% compared to a nontargeting control. Detailed analyses included histological, ultrastructural, functional (water content, water evaporation, dye penetration) proteomic and lipid mass spectrometry. Over-expression of *EMSY* was achieved by viral vector transfection of primary keratinocytes, to compare the effect with *EMSY* knockdown.

Main findings

EMSY acts as a transcriptional repressor in human keratinocytes. Knockdown leads to a marked change in skin organoid phenotype, including acceleration of barrier formation and upregulation of multiple aspects of skin barrier function. Over-expression in cells shows opposite effects. EMSY knockdown is therefore a novel therapeutic target for eczema treatment and/or prevention.

[5] Longitudinal latent class analysis identifies distinct subgroups of paediatric eczema in two independent populations

Rationale

Atopic eczema is a highly heterogeneous disorder, with variation in age of onset, chronicity, severity and comorbidities. One approach to sub-classify eczema cases is to define ‘atopic’ (extrinsic) and ‘non-atopic’ (intrinsic) forms, based on the presence or absence of IgE elevation. It is well recognised that the majority of eczema cases present within the first two years of life and most resolve during childhood,¹⁵ but some persist into adulthood.²¹² The heterogeneity of longitudinal disease course is relatively un-studied, but it could offer valuable insight into eczema sub-phenotypes.

Latent class analysis applied to longitudinal birth cohorts with detailed phenotype data

Longitudinal latent class analysis (LLCA) is a statistical technique which can be used to model distinct subgroups within a dataset to identify different longitudinal patterns of disease, where the aim is to identify distinct subgroups in longitudinal multivariate categorical data. LLCA is somewhat similar to cluster analysis, but it allows for assignment based on mathematical probability, rather than definitive partitioning of individuals into classes. Starting with a single latent class, additional classes are added until measures that estimate the degree of model fitting are optimised. Statistical criteria (including a low Bayesian Information Criterion) are used to determine the optimal number of classes within the data.

This work applied LLCA to data from two independent longitudinal cohort studies: The Avon Longitudinal Study of Parents and Children (ALPSAC) in the UK and Prevention and Incidence of Asthma and Mite Allergy (PIAMA) in The Netherlands.

Main findings

The independent cohorts gave remarkably similar results, offering confidence in this mathematical modelling approach. Six distinct subtypes of eczema were defined (one of which was essentially unaffected), including a previously unrecognised class with peak onset at the age of 5-6 years, associated with male sex and asthma. The classes of early onset and persistent or late-resolving eczema showed the strongest association with eczema risk variants from GWAS.

[6] Genome-wide comparative analysis of atopic dermatitis and psoriasis gives insight into opposing genetic mechanisms

Comparing and contrasting two common inflammatory skin diseases

Eczema and psoriasis are highly prevalent inflammatory skin disorders but they very rarely co-exist.²¹³ Clinically, these diseases have similarities as well as differences. They are both erythematous and scaly; both sometimes itchy; both may be generalised, but eczema is characteristically flexural whilst psoriasis affects some flexures (*e.g.* umbilicus, groin, axillae and retro-auricular) but is classically extensor; eczema has atopic co-morbidities whilst both share the co-morbidities of cardiovascular disease^{30,214,215} and psychological/psychiatric burden.^{32,216} On a genome-wide level the two diseases show considerable overlap in risk loci, including the epidermal differentiation complex (chromosome 1q21.3), the Th2 locus control region (chromosome 5q31.1) and the major histocompatibility complex (chromosome 6p21-22).

These observations provide the rationale for a genome-wide comparative analysis, to investigate shared and opposing mechanisms leading to eczema or psoriasis.¹⁹⁷

Collaborative work and statistical method development

Genome-wide genotype data were obtained on samples from six case-control cohorts (three each of eczema and psoriasis), totalling 2,262 eczema cases, 4,489 psoriasis cases and 12,333 controls. Meta-GWAS was performed on each disease using standard methodologies. Novel meta-analysis techniques were developed, in collaboration with an expert in statistical genetics (Heather Cordell, Newcastle upon Tyne, UK) and applied to the datasets to compare and contrast genetic effects between the two diseases.

Main findings

Opposing risk variants were identified within the epidermal differentiation complex, the Th2 locus control region and the major histocompatibility complex. Previously unreported pleiotropic alleles with opposing effects on eczema and psoriasis risk were also identified in *PRKRA* and *ANXA6/TNIP1*. In contrast, there were no shared loci with effects operating in the same direction on both diseases. Atopic eczema and psoriasis therefore appear to have distinct genetic mechanisms with opposing effects in pathways influencing epidermal differentiation and immune response.

CHAPTER FOUR: Critical appraisal

Contributions to the field of knowledge

Each of the six publications featured in the thesis have contributed knowledge to the field of eczema genetic research. The main contributions are summarized in turn below.

First global transcriptomic analysis of atopic skin¹⁰¹

Patients with atopic eczema have a generalised abnormality in skin biology that is not restricted to the areas affected by eczematous inflammation. Atopic skin demonstrates a greater transepidermal water loss and greater penetration of external allergens and irritants.²¹ A subset of patients with eczema also show greater susceptibility to bacterial¹⁵ and viral²¹⁷ infection. Furthermore, *FLG* haploinsufficiency is a specific risk factor for eczema herpeticum,²¹⁸ a complication in which herpes simplex infection spreads rapidly in areas of skin affected with active eczema, causing a severe and sometimes life-threatening condition.³⁶ The molecular mechanisms underpinning these abnormalities are not fully understood.

I was fortunate to have the opportunity to collect, with ethical approval, skin biopsies from a series of children who had been cared for in Our Lady's Children's Hospital Crumlin, Dublin, for their lifelong atopic eczema. These precious samples warranted very careful analysis. At that time, previous studies had investigated the skin transcriptome using microarray and quantitative PCR. These had identified abnormalities in epidermal differentiation,^{219,220} inflammation^{221,222} and lipid structures.²²³ However, microarray analysis is limited by the requirement to preselect genes for inclusion in an array, hence RNA-sequencing has become the method of choice for global transcriptome analysis. Single-molecule direct RNA sequencing (DRS)^{224,225} was used as the optimal methodology for quantification of the small amounts of mRNA available from the paediatric skin biopsies. 'Uninvolved' skin (*i.e.* skin that appears normal on clinical examination) was studied to avoid the confounding effects of inflammation and secondary infection. My work represented the first comprehensive analysis of primary molecular abnormalities in atopic skin.

One striking finding was the global effect of *FLG* genotype on skin transcriptome, illustrating the central role that filaggrin plays in epidermal biology. Analysis of the

whole dataset (all cases compared to all controls) showed significantly differentially expressed genes, when classified by gene ontology (GO) in keeping with previous micro-array studies – an important proof-of concept. However, GO analysis stratified by *FLG* genotype revealed key differences in *FLG* mutant and wild-type cases compared with controls: *FLG* wild-type cases showed dysregulation of genes involved with lipid metabolism and upregulation of extracellular matrix genes whilst *FLG* mutant cases displayed upregulation of a functional network of proteins in the type 1 interferon-mediated defence response. I have speculated that this network of ‘defence response’ might relate to the dysfunctional cutaneous response to viral infection in patients with eczema, or it may represent the reduced threshold for inflammation that is apparent clinically in many atopic patients.^{226,227}

Together these transcriptomic analyses provided insight into the mechanistic pathways in atopic skin, which are both dependent and independent of *FLG* genotype, including the importance of extracellular space, lipid metabolic pathways, apoptotic pathways, innate and acquired immunity. Together these findings contribute to the future development of stratified and personalised medicine.

***FLG* genotype has no substantial effect on photo-response in normal skin¹⁹⁴**

This study was designed to address a widely-quoted hypothesis stating that the filaggrin breakdown product *trans*-UCA contributes to skin photoprotection by its absorption of UVB to produce *cis*-UCA, which in turn has immunomodulatory effects.⁷² There is evidence of these effects in human cells *in vitro* and in mice *in vivo*, but no previous studies in human skin *in vivo*. The response of skin to UV radiation ranges from erythema and oedema, to blister formation and necrosis. The molecular mechanisms are complex and multifaceted, but they include DNA damage and apoptosis. This study aimed to use erythematous response as a quantitative parameter of clinical relevance; clinically-relevant wavebands and doses of UV radiation were used, including the UVA and UVB wavelengths used for eczema treatment.

Somewhat surprisingly, there was no difference in erythematous sensitivity between the *FLG* heterozygous and wild-type volunteers, even in the UV wavelengths known to be absorbed by UCA as it undergoes *trans* to *cis* isomerisation. Importantly, our post-hoc power estimation showed that the sample size (71 individuals, including 10

FLG heterozygotes) was sufficient to exclude an effect size of >1.8-fold difference in MED; a smaller effect could not be excluded. However, there was no differential effect in the wavebands expected to be absorbed by *trans*-UCA and those not known to be absorbed by this photochemical, suggesting that the hypothesized mechanism of photoprotection does not have an impact on erythral sensitivity.

This work represents an important negative finding. The fact that observations of UVB-induced damage in murine and *in vitro* models have not been supported by clinical data suggests that different mechanisms lead to cutaneous erythema *in vivo*. This is perhaps un-surprising, given the more complex and multifaceted mechanisms of photoprotection *versus* photodamage in human skin *in vivo* compared to *in vitro*. Epidemiological studies by other research groups have shown an increase in actinic damage in patients with *FLG* null mutations²²⁸ and it is possible that the photoprotective effect of the *FLG* wild-type genotype might be attributable to a mechanical filtering of UV by the stratum corneum, rather than by photo-immunosuppression. However, it is not clear whether the observational association of *FLG* mutant genotype and actinic damage is the result of filaggrin deficiency or confounding factors, for example sun-seeking behavior by patients with chronic eczema or their use of immunosuppressant medications.

Another important impact of these findings is that they demonstrate the need for future work to define predictors of patient response to phototherapy for eczema. The treatment dose of UV radiation is currently based on reported skin phototype and cutaneous response to MED testing. There are no reliable predictors of response to UVA or UVB phototherapy for atopic eczema. A greater understanding of factors predicting response would clearly be superior to the current approach of using a therapeutic trial, with the inherent risk in UV exposure.

Skin organoid models allow detailed functional and molecular assessment of eczema GWAS loci^{117,195}

Multiple independent research groups have developed skin organoid models with important technical differences, including the presence or absence of a model dermis and the source(s) of keratinocytes for model epidermis. Immortalised cell lines or primary human keratinocytes from patients with normal skin (from foreskin or other surgical waste tissue, pooled or single-donor), active eczema or *FLG* mutant skin

samples have each been used. This diversity of modelling has produced a range of findings, summarised by Niehues *et al.*¹¹³ and listed in a review that I co-authored.⁶⁵ The somewhat conflicting findings in skin barrier assessment *in vitro* may be accounted for, at least in part, by methodological differences in organoid culture as well as details with respect to the measurement of barrier function. The duration of dye exposure, for example, may affect sensitivity to detect barrier impairment. In our model, the immature organoids showed some limited evidence of barrier impairment with *FLG* knockdown, but the mature organoids showed no difference in transepidermal water loss, water content or dye penetration. The concordance of these three sensitive measurements gives confidence to the findings.

Our work used a model developed and optimised for barrier assessment, informed by a detailed protocol shared by Mildner *et al.*¹¹⁴ I chose to use primary human keratinocytes from normal skin donors, allowing matched controls and technical replicates, in addition to multiple biological replicates to allow for inter-individual variation.

The filaggrin-deficient phenotype *in vitro* had previously been studied in detail.¹¹⁶ Elias *et al.* used pooled foreskin keratinocytes and shRNA knockdown, in an epidermal-only model; each of these aspects are likely to reduce, to some degree, the physiological relevance of the model. In contrast, the siRNA knockdown in my work allowed the use of low passage keratinocytes which produce organoid histology that more closely resembles skin *in vivo*. The use of single donor tissue also allowed separate biological replicate experiments for comparison and the matching of keratinocytes and fibroblasts for the epidermal and dermal compartments.

My work added greater detail in the mass spectrometry proteomic analysis: Fractionation to increase the sensitivity allowed detection of more than 8000 proteins compared to 1640 proteins by Elias *et al.*¹¹⁶ The findings from my work are complimentary to those of Elias *et al.* and have replicated the finding that filaggrin deficiency affects the abundance of multiple proteins of relevance to eczema pathophysiology, even in the absence of inflammation. However, the greater detail and closer physiological relevance of my work offered additional insights, including evidence of mechanisms contributing to immune and neurological, as well as structural, aspects of skin barrier dysfunction.

This work *in vitro* is complimentary to previous studies of eczema skin biopsies in my own work described above¹⁰¹ and carried out by other groups.^{100,229} The nature of our model and analysis of isolated epidermal tissue means that the mechanisms are keratinocyte-specific and cannot be confounded by differential components of immune cells, neural tissue, blood vessels *etc* that may be present in whole skin biopsies to differing degrees. Furthermore, the skin organoid model is important in that it provides an opportunity to study keratinocyte-specific mechanisms in isolation from other cell types. This gives unique insight into skin-specific genetic mechanisms as well as opportunities for the development of treatments which may be most effective when applied topically in direct contact with keratinocytes. My work was published in *Wellcome Open Research*¹¹⁷ because this journal allowed sharing of detailed methodology as well as data. The detailed proteomic data have been shared in raw and processed form using the open access platform provided by the European Bioinformatics Institute (EMBL-EBI) PRIDE (<https://www.ebi.ac.uk/pride/>) as a resource for the field.

Mass spectrometry lipidomic analysis of the filaggrin-deficient skin organoid model was also carried out, using spiked-in standards to aid identification of ceramide subsets. This work produced findings in keeping with previous lipid analyses *in vitro*^{230,231} and *in vivo*²³² providing further proof of concept that the skin organoid provides an effective recapitulation of skin barrier physiology.

I next applied the organoid model to the investigation of a novel eczema candidate gene – *EMSY* – which had not previously been studied in skin. siRNA knockdown of *EMSY* led to a marked phenotype change. An acceleration of the skin barrier formation was observed, with upregulation of multiple proteins involved in skin differentiation, as well as lipid components of the stratum corneum. These findings demonstrate an important role for *EMSY* in transcriptional regulation and skin barrier formation.

To investigate the relevance for clinical disease, I studied *EMSY* expression in eczema and control skin biopsies. Here, the intensity of nuclear expression at the basal layer and throughout the epidermis, was a marker of active eczema. In subsequent work, I have shown a correlation between the risk SNP genotype and *EMSY* expression in primary keratinocytes (unpublished data) which further validates the link between *EMSY* and the GWAS risk locus.

Together these findings strongly support EMSY inhibition as a novel therapeutic approach for atopic eczema. Recent therapeutic advances have effectively targeted atopic inflammation and therapies specifically aiming to increase skin barrier function are not currently available. I am now working in collaboration with experts in the fields of high-throughput screening and drug discovery to assess the feasibility of targeting EMSY as a treatment for eczema and other skin barrier defects.

There are distinct subgroups of atopic eczema within the human population¹⁹⁶

Atopic eczema is a very diverse phenotype in clinical appearance, age of onset, co-morbidities, response to therapy and disease trajectory. Loss-of-function mutations in *FLG* are known to be a risk factor for early-onset, persistent and severe disease; the presence of elevated total and specific IgEs are also associated with more severe disease, but this may be an epiphenomenon, rather than a true marker of pathophysiological process. There are well-recognised clinical subsets of eczema, with remission in infancy, later childhood, or evolution into a life-long chronic disease. However, it was not clear whether the observed differences represent a continuum, or distinct subgroups, since the definition of longitudinal disease subsets had not previously been studied.

Collaboration with two large, well designed, longitudinal birth cohort studies allowed the technique of longitudinal latent class analysis to be used to test for distinct subgroups. This was the first application of LLCA to atopic eczema. Adjustment of the modelling parameters defined 6 groups as providing the optimal balance between fitting and over-fitting the data. Findings from each of the independent populations gave remarkable replication, supporting the validity of these latent classes.

The majority of eczema cases in the birth cohorts were, as expected, in a large class of early onset and resolving disease. The cases with *FLG* mutations (and other GWAS risk SNPs) were most strongly associated with classes of persistent or late-resolving eczema; this was also as expected from previous knowledge. Novel findings included evidence for distinct subgroups of atopic eczema in childhood, and the presence of a previously unrecognised class with onset at 5-6 years of age, associated with atopic asthma and male sex.

The finding of distinct latent classes provides support for the future application of personalised medicine tailored to the specific subsets of eczema. However, this LLCA is observational. Further research is needed to apply our findings for the prediction of eczema trajectory (rather than the retrospective definition supplied by the longitudinal data) before pro-active personalised care can be made available.

Eczema and psoriasis show opposing genetic mechanisms in epidermal differentiation and immune response¹⁹⁷

It has long been recognised that eczema and psoriasis rarely co-occur, but the molecular mechanisms for this exclusivity are not well defined. It is a striking observation that atopic eczema and psoriasis share genetic risk loci, and these have been attributed to both immune and skin barrier mechanisms.²³³ Previous work investigated whether SNPs reported to be associated with psoriasis were also associated with atopic eczema.²³⁴ Of the 13 risk variants in the overlapping loci reaching statistical significance, approximately two-thirds showed opposing effects on eczema and psoriasis (consistent with the diseases being mutually exclusive) whilst one-third of variants contributed risk to both diseases. This may be representative of the shared features of the two inflammatory skin diseases.²³⁴

My work took a more systematic exploratory approach, using statistical analysis to compare and contrast genetic risk for eczema and psoriasis across the whole genome. Our results confirm shared regions of genetic risk on chromosomes 1, 5 and 6, representing the epidermal differentiation complex, Th2 locus control region and the major histocompatibility complex (MHC). Within these regions there were variants with shared and opposing effects on eczema and psoriasis, but the variants increasing risk of both diseases were not statistically significant, emphasising the divergence of these conditions.

There were 6 loci associated with both diseases (with genome-wide significance) and each showed opposing effects. A variant within the antisense transcript *FLG-AS1* may mediate opposing effects on epidermal differentiation through transcriptional regulation of nearby genes, as has been described for other long non-coding RNA species.^{235,236} Opposing effects at 5q31.1 are within *RAD50* and *IL13*, therefore likely to reflect the divergent immune signalling in eczema *versus* psoriasis. Two other loci (2q31.2, 5q33.1) had not previously been reported as showing co-association with

eczema and psoriasis. The pleiotropic alleles in these two loci with opposing effects on eczema and psoriasis risk lie in exon 6 of *PRKRA* and in an intergenic region in linkage disequilibrium with *ANXA6* and *TNIP1*. *PRKRA* encodes protein kinase interferon-inducible double-stranded RNA-dependent activator (PACT), a cellular dsRNA-binding protein originally identified as a binding partner and activator of PKR in response to extracellular stress.²³⁷ More recently, it has been shown to be an essential factor in the PKR-independent initiation of RIG-I-induced antiviral response.²³⁸ This may be of relevance to the differential susceptibility of individuals with eczema and psoriasis to viral skin infections.²³⁹ *ANXA6* encodes a calcium-dependent membrane and phospholipid binding protein that is upregulated in atopic skin compared to control skin but expression is decreased in skin with active psoriasis. Since calcium signalling is important in epidermal differentiation this may contribute to differential susceptibility to the inflammatory skin diseases, but further fine mapping and functional testing is required. *TNIP1* is involved in TNF signalling and regulation of the transcription factor NF- κ B and it has previously been implicated in psoriasis risk²⁴⁰ but not eczema. *TNIP1* shows increased expression in both eczema and psoriatic lesions compared to control skin, which supports this mechanism as an opposing effect in these disorders. It is tempting to speculate that the reported observations of eczematous eruptions or paradoxical psoriasis developing in patients receiving anti-TNF biologic therapies²⁴¹ and psoriatic eruptions in response to dupilumab for eczema²⁴² may all be manifestations of the balance that is required in healthy skin to maintain an immune homeostasis to avoid either eczema or psoriasis. Greater understanding of this immunological balance may be applied to therapeutic optimisation in the future.

Looking outside Dermatology, the statistical analysis methods developed in the conduct of this study are likely to be applicable to the investigation of the genetic basis of other complex traits with overlapping and distinct clinical features.

Impact on clinical guidelines and randomised controlled trials

The role of *FLG* null mutations in causing dry skin, barrier dysfunction and eczema has informed the development of therapeutic guidelines. The National Institute for Health and Care Excellence (NICE) guidelines state: '*Atopic eczema often has a genetic component that leads to the breakdown of the skin barrier. This makes the*

skin susceptible to trigger factors, including irritants and allergens, which can make the eczema worse'.²⁴³ The NICE guideline on treatment emphasises the importance of emollient use to improve skin barrier function, now a key quality statement: '*Children with atopic eczema are prescribed sufficient quantities (250-500 g weekly) from a choice of unperfumed emollients for daily use.*' The Scottish Intercollegiate Guidelines Network (SIGN) also quote filaggrin research in their rationale for a guideline: '*...defects in epithelial barrier function arising from abnormalities in structural proteins such as filaggrin [make] the skin both excessively permeable and more prone to damage from environmental irritants and allergens.*'²⁴⁴ This new understanding has been conveyed in undergraduate and postgraduate teaching as well as lay health education including the NHS educational web pages 'NHS inform' and 'NHS choices'.

The strong effect of *FLG* null genotype has also been used for patient stratification in randomised controlled clinical trials, including therapeutic silk clothing¹⁹⁰ and emollients for eczema prevention.²⁴⁵

Strengths and weaknesses

Many of the strengths and weaknesses of my published research have been discussed in relevant sections above; additional key points will be summarised here.

Atopic skin transcriptome analysis

A major strength of direct RNA sequencing (DRS) is that it offers accurate quantification of polyadenylated RNA, avoiding the bias that is introduced by the steps of reverse transcription, ligation, and amplification in other forms of RNA sequencing. In addition, DRS provides information on the DNA strand from which the mRNA is transcribed, allowing more accurate alignment of reads to genes in complex regions. The disadvantages of DRS are that relatively short reads are produced, hence the technique is better for accurate quantification than novel sequencing. However, the main aim of my analysis was to quantify the transcriptome in the most sensitive, accurate and comprehensive way, rather than to detect novel transcript sequences, therefore DRS was the optimal method for this detailed study of tissue-specific gene expression data from small amounts of tissue.

Another strength of the skin transcriptome study was the very thorough *FLG* genotyping by full sequencing, rather than selected mutation analysis. This identified several mutations that would otherwise have been overlooked. The application of *FLG* genotype – known to have a functional effect on skin biology – for stratification was a strength allowing more information to be leveraged from the large dataset. This is illustrated by the differing but complimentary findings obtained from analysis of the whole dataset compared with *FLG* genotype subgroups.¹⁰¹

A weakness of the skin transcriptome analysis was that I did not have sufficient tissue to allow protein quantification to investigate correlation with mRNA levels. This was a limitation because of the biopsy size (3 or 4mm diameter punch) which was the maximum that could be requested from our paediatric patients. I made the decision to use the entire sample for optimal RNA extraction, since my pilot data from similar sized skin samples showed that attempts to bisect the sample or protocols to extract RNA and protein simultaneously compromised the quality and quantity of RNA.

Erythema response to UV radiation

A major strength of this study was the use of clinically-relevant wavebands and physiological doses of UV radiation. A relative weakness was the sample size with detailed phototesting data, meaning that the study was only powered to detect a modest-large effect size (>1.8-fold difference in MED). Another possible weakness of the study was the lack of *FLG* homozygous or compound heterozygous individuals; this was not unexpected since the prevalence of *FLG* -/- is only ~1:100 within the normal population. We therefore have not excluded the possibility that *FLG* homozygous (or compound heterozygous) subjects might show greater erythema sensitivity than wild-type subjects. However, *FLG* null heterozygosity has a significant effect on filaggrin expression *in vivo*,^{101,126} therefore I would anticipate an effect to be detectable in this study (within the parameters of the posthoc power calculation) reducing the likelihood of a false negative finding.

Organoid modelling of genetic effects in skin

The skin organoid model optimised in my laboratory uses low passage primary human keratinocytes and donor-matched primary fibroblasts; this is a major strength

in obtaining as near to physiological conditions as possible *in vitro*. Other important cell types, notably resident and circulating immune cells of the haematopoietic lineage are absent; supportive and adnexal structures including blood vessels, neurons, sweat glands *etc* are also absent. The reductionist model may clearly be a weakness but it can also be viewed as a strength. My approach focuses on epidermal keratinocytes because there is clear evidence of specific genetic effects in keratinocytes in disorders of cornification with atopic features^{78,195,246} and barrier epithelial cells are crucial in determining the outcome of allergen-encounter.²⁴⁷ Keratinocyte biology is likely to play a central role in eczema risk and resolution. Studying keratinocytes in a tissue model without other immune cells offers the opportunity to identify initiating mechanisms in eczema, as well as keratinocyte-specific contributions to barrier function.^{78,195,248} The potential weaknesses in using the keratinocyte and fibroblast organoid model are further circumvented by validation of key findings in whole skin biopsies from eczema patients.^{117,195}

Longitudinal latent class analysis defining eczema subgroups

A major strength of this study is the use of data from two large and complimentary longitudinal epidemiological studies. Despite the large sample size, a limitation inherent in latent class analysis is that the dataset is divided into smaller subsets, which lowers the statistical power to detect association. The similarity in case definitions, using prospective questions to capture diagnostic features of eczema from infancy to later childhood and the similar high frequency of eczema in ALSPAC and PIAMA are strengths. However, these studies were limited in their inclusion of data relating to atopic traits, comorbidities and important environmental factors. Another limitation inherent in LLCA was the fact that the analysis is not designed to test for causality of direction of effect. This is pertinent because some of the risk factors in this study did not unequivocally precede the onset of disease. Therefore further work is needed to investigate causality (discussed below).

Comparative analysis of eczema and psoriasis

A major strength of this work was the large sample sizes, but weaknesses included the different platforms used for genotyping in different cohorts, meaning that imputation and careful integration of data were needed. These steps may have introduced bias. A further limitation in the interpretation and application of key

findings is that genome-wide analysis identifies loci and variants in association with disease, but not a mechanism of effect. Further work is therefore needed to fine-map and validate key findings by demonstrating functional effects *in vitro* or *in vivo*. Work is now on-going in my lab to investigate one of the loci showing an opposing effect within the *FLG-AS1* transcript. Preliminary (unpublished) findings show an effect of *FLG-AS1* on the expression levels of *FLG* and *FLG2*.

Genetic diversity

The lack of ethnic diversity in all six publications arose because of study design, in which genetic homogeneity was needed to allow screening for *FLG* null mutations that are prevalent in white European populations and to reduce inter-individual variation to some extent. However, this lack of diversity is a weakness that has been highlighted as a widespread problem in the field of genetic research.¹⁸² An increasing number of studies are investigating eczema genetic risk in more ethnically diverse populations²⁴⁹ including African people-groups.^{94,250-253}

Sex-differences are also an important consideration in genetic research. Participant sex has been matched or controlled for throughout my work wherever possible. An exception is in the skin organoid work, since the primary cells for these studies are harvested from predominantly female skin, reflecting the plastic surgical procedures (breast and abdominal reduction surgery) that most frequently supply excess tissue. Ideally future work would test for replication using XY cells and tissues.

Complimentary approaches

A variety of complimentary experimental approaches are used in my work and this is, I believe, an important strength. Complimentary approaches offer a more comprehensive understanding of the very complex and diverse disease, atopic eczema. The integration of different perspectives obtained from clinical observation, epidemiological and population-based studies, molecular and genetic analyses has provided opportunities to inform and direct future research. For example, the skin transcriptome findings from DRS have informed the *FLG* knockdown *in vitro* and clinical studies *in vivo*; GWAS findings led to assessment of *EMSY* in skin models *in vitro* and validation of *EMSY* expression in clinical biopsy samples, leading to a phenotypic screen for drug discovery.

Outstanding questions and future perspectives

Despite rapid progress in the field of eczema genetic research there remain important gaps in knowledge and understanding.

Molecular mechanisms attributable to filaggrin

Some fundamental questions remain to be answered in relation to the filaggrin molecule, profilaggrin and the constituent amino acids with respect to how filaggrin deficiency leads to eczema and other atopic diseases. More specific understanding of the relative importance of different aspects of the profilaggrin-filaggrin-amino acid pathways and their control mechanisms could allow for more effective targeting of this central pathomechanism for treatment and/or prevention. My work and others' has shown that filaggrin plays a role in skin biology beyond the mechanical barrier function of filament aggregation to susceptibility to infection, threshold for inflammation, control of apoptosis and neurological abnormalities. Effective therapeutic application may require specific targeting of one or more of these aspects of skin biology.

Insights from GWAS

Multiple GWAS studies and meta-analyses have identified risk loci, emphasizing the importance of genetic risk mechanisms controlling both the skin barrier and immune responses in eczema, but for the majority of risk loci the mechanisms leading to atopic skin inflammation remain unknown. Furthermore, many of the loci are intergenic, therefore detailed molecular studies, carried out in cells and tissues of relevance to eczema are needed to characterise the relevant functional effects. The threshold for statistical significance is necessarily stringent in GWAS because of the extreme multiple testing that occurs (**Figure 4**). Larger GWAS studies, including hundreds of thousands of cases and controls could reveal additional risk loci, but each new effect size is likely to be small. GWAS findings to date account for <20% of AD heritability⁹² and even with the additional risk attributed to protein-coding variants, ~70% of heritability remains to be explained²⁵⁴. Considerable further work is therefore needed to fully understand individual risk and functional effects.

In addition to the main GWAS 'hits', multiple gene–gene and gene-environment interactions that are likely to contribute to the heritability that remains to be

explained in atopic eczema (**Figure 4**). Gene-gene interaction analysis is statistically challenging because of the issues of multiple testing and similarly gene-environment interactions, whilst likely to be of importance in eczema, are challenging to detect on a genome-wide level. These mechanisms therefore require alternative, more targeted functional assessment. A clearer understanding of gene-environment interaction is needed in order to explain the significant rise in incidence of atopic disease in industrialized nations over the recent decades. This will require careful epidemiological study of human populations, but the use of mouse models of atopic disease will also be useful in allowing experimental manipulation of both genetic and environmental effects. Gene-environment interactions are key to future preventative strategies, with the ultimate aim of preventing atopic eczema and halting the atopic march.

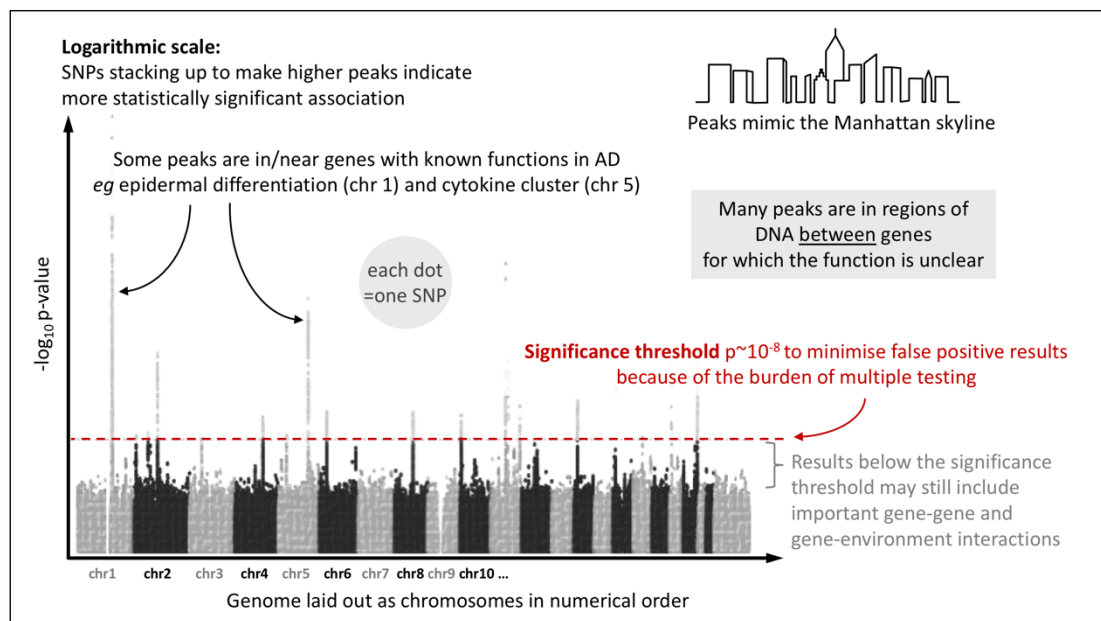


Figure 4. Annotated Manhattan plot showing information displayed and outstanding questions.

A Manhattan plot is the conventional method for displaying results from GWAS. Each SNP is represented by a dot on the plot, and its position is determined by genomic location and a statistical test of association with the trait of interest. Figure reproduced from an invited commentary that I have written, currently under review for *J Invest Dermatology* 2020.

Causal effects

Population genetic studies including GWAS and LLCA identify association but do not test for causal effects. Causality can be tested *in vitro* using skin organoid models (as described above), but this detailed work is very time-consuming. An alternative statistical approach is Mendelian randomisation (MR), a technique that uses genetic risk variants, single or groups of SNPS from GWAS, as a proxy for phenotype or exposures. Genetic variants are inherited randomly at conception, therefore they are less prone to the confounding and reverse causation which limit the usefulness of observational epidemiology.²⁵⁵ MR studies in eczema have previously investigated causal links with prenatal alcohol exposure²⁵⁶ and vitamin D levels,²⁵⁷ each of which have no causal effect on eczema. Another approach has combined MR and multiple-trait colocalization to define cell-specific inflammatory drivers of autoimmune and atopic disease.²⁵⁸ A recent MR study has shown that there is a causal effect of obesity on psoriasis, but conversely psoriasis does not cause an increase in obesity.²⁵⁹ We have recently completed a similar MR analysis to investigate a causal effect of obesity on eczema, or *vice versa* (manuscript in press, *J Allergy Clin Immunol*).

Therapeutic targets

It is known that drugs targeting molecules or pathways informed by human genetic studies have a greater chance of clinical success than targets defined using other experimental or theoretical approaches.²⁶⁰ GWAS hits and variants in protein-coding regions have identified multiple proteins in the IL-13 pathway, all of which have been successfully targeted in novel AD treatments.²⁵⁴ In contrast, specific molecular targets to improve skin barrier function remain elusive and, as discussed above, in this respect *EMSY* is a particularly promising discovery. The distinct genetic mechanisms in atopic eczema and psoriasis include opposing effects in pathways influencing epidermal differentiation and immune response, suggesting that both may need to be targeted simultaneously to achieve optimal disease control. Further investigation of disease mechanisms will offer the opportunity to understand not only inflammatory skin disease but also the substantial burden of co-morbidities. Translational genomics, drug development and personalised medicine are closely inter-dependent and will progress together.^{61,261} Dermatological research is poised to be at the forefront of these exciting developments in clinical care.

Another area of intensive research interest is the complex interaction of skin and gut microbiome with cutaneous inflammation.²⁶² These studies have been made possible by modern sequencing technologies to circumvent the need for selective bacterial cultures.²⁶³ The approach has illustrated a reduction in microbial diversity in active eczema in addition to the well-established over-growth of *S aureus*.²⁶² Further work is needed to match genetic risk effects in human skin with microbial diversity or overgrowth²³² and to date these interactions have not been targeted for therapy.

Can eczema be prevented?

There is considerable interest in the possibility that eczema and other atopic diseases may be amenable to primary prevention through targeted intervention early in life. Hope was raised by two independent studies which both showed an ~50% reduction in eczema prevalence in high-risk infants treated with intensive emollient therapy.^{264,265} However, a recent more definitive study (the BEEP study, Barrier Enhancement for Eczema Prevention), including 1394 newborns in a randomised controlled study, has shown no protective effect.²⁴⁵ This study included planned subgroup analysis based on *FLG* genotype (for which I co-ordinated the genotyping). It could be hypothesised that either filaggrin-deficient or filaggrin-replete skin would benefit more from emollient therapy: filaggrin-deficient because this skin stands to derive greater benefit from the hydration provided by emollient therapy; filaggrin-replete because these infants' skin does not have an inherent predisposition to inflammation. The fact that neither of these genetic subgroups showed a protective effect from emollient application provides further support for the whole study's negative result.

A similar study from Sweden, including 2397 newborn infants has replicated the finding that emollient therapy does not protect against atopic eczema.²⁶⁶ These are both well-designed, sufficiently powered and carefully conducted studies. Possible reasons behind the negative results include: true lack of effect in emollient for eczema prevention, since the independent studies gave strikingly similar results; inadequate emollient application, although there was adherence to emollient use in >70% of the intervention group in the BEEP study; delayed commencement, possibly after a critical early window for eczema initiation; or an insufficiently complex intervention to improve skin barrier.

Interestingly, analysis of side effects and secondary effects of emollient showed some evidence of increased skin infection (adjusted incidence rate ratio 1.55 (95% confidence interval 1.15 to 2.09) and a non-significant increase food allergy.²⁴⁵ Therefore intensive emollient application may not be entirely harm-free. Further work is needed to identify the most effective way to improve skin barrier early in life and assess whether this can safely be used to prevent eczema or other atopic diseases.

Concluding remarks

The work comprised in these six selected publications illustrates a program of research that has contributed substantially to understanding genetic risk mechanisms in atopic eczema. The field has shifted from a focus on atopic inflammation to a broader understanding of skin barrier-immune dysregulation as a complex interplay, with multiple genetic as well as environmental effects. Much has been learned from genetic studies, allowing a detailed interrogation of the primary mechanisms in atopic skin disease and there is great potential for this knowledge to be applied in future therapeutic development.

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APPENDIX I: Statement of authorship

Publication	Proportion and nature of contribution
Cole C, Kroboth K, Schurch NJ, Sandilands A, Sherstnev A, et al., Watson RM, McLean WHI, Barton GJ, Irvine AD and Brown SJ. Filaggrin-stratified transcriptome analysis of paediatric skin identifies mechanistic pathways in atopic dermatitis. <i>J Allergy Clin Immunol.</i> 2014; 134(1):82-91.	I contributed to the conception, design and conduct of this study; I carried out all practical procedures and sample processing; I guided and supervised the statistical analysis of DRS data; I led the interpretation and validation of findings; I wrote the first draft of the manuscript and coordinated all edits before submission for publication.
Forbes D, Johnston L, Gardner J, MacCallum SF, Campbell LE, Dinkova-Kostova A, McLean WHI, Ibbotson SH, Dawe RS and Brown SJ. Filaggrin genotype does not determine the skin's threshold to UV-induced erythema. <i>J Allergy Clin Immunol</i> 2016;137(4):1280-2.	I contributed to the conception, design and conduct of this study; I contributed to the statistical analysis of data and interpretation of the findings; I wrote the first draft of the manuscript and coordinated all edits before submission for publication.
Elias MS, Wright SC, Nicholson WV, Morrison KD, Prescott AR, Ten Have S, Whitfield PD, Lamond AI and Brown SJ. Functional and proteomic analysis of a full thickness filaggrin-deficient skin organoid model. <i>Wellcome Open Res.</i> 2019, 4:134.	I designed this study and contributed to the practical work; I guided and supervised all aspects of the analysis and led the interpretation and validation of findings; I wrote the first draft of the manuscript and coordinated all edits before submission for publication.
Elias MS, Wright SC, Remenyi J, Abbott JC, Bray SE, Cole C, Edwards S, Gierlinski M, Glok M, McGrath JA, Nicholson WV, Paternoster L, Prescott AR, Have ST, Whitfield PD, Lamond	I designed this study and contributed to the practical work; I guided and supervised all aspects of the analysis and led the interpretation and validation of findings; I wrote the first draft of the

<p>AI and Brown SJ. EMSY expression affects multiple components of skin barrier with relevance to atopic dermatitis. <i>J Allergy Clin Immunol</i>. 2019 Aug;144(2):470-481.</p>	<p>manuscript and coordinated all edits before submission for publication.</p>
<p>Paternoster, L Savenije OEM, Heron J, Evans DM, Vonk JM, Brunekreef B, Wijga AH, Henderson AJ, Koppelman GH and Brown SJ. Identification of atopic dermatitis subgroups in children from two longitudinal birth cohorts. <i>J Allergy Clin Immunol</i> 2018;141(3):964-971.</p>	<p>I contributed to the conception, design and conduct of this study; the latent class analyses were carried out by collaborators (Paternoster and Savenije) with my guidance; I led the interpretation of findings; I co-wrote the first and final drafts of the manuscript with major contributions to the clinical interpretations and discussion.</p>
<p>Baurecht H, Hotze M, Brand S, Büning C, Cormican P, Corvin A, Ellinghaus D, Ellinghaus E, Esparza-Gordillo J, Fölster-Holst R, Franke A, Gieger C, Hubner N, Illig T, Irvine AD, Kabesch M, Lee Y AE, Lieb W, Marenholz I, McLean WHI, Morris DW, Mrowietz U, Nair R, Nöthen MM, Novak N, O'Regan GM, PAGE consortium, Schreiber S, Smith C, Strauch K, Stuart PE, Trembath R, Tsoi LC, Weichenthal M, Barker J, Elder JT, Weidinger S, Cordell HJ and Brown SJ. Genome-wide comparative analysis of atopic dermatitis and psoriasis gives insight into opposing genetic mechanisms. <i>Am J Hum Genet</i>. 2015; 96(1):104-20.</p>	<p>I conceived this study and contributed to the design; novel methods of statistical analysis were designed by Cordell and analysis was conducted principally by Cordell and Baurecht with discussion and guidance from Weidinger and myself; I led the interpretation and discussion of findings; I wrote the first draft of the manuscript and coordinated all edits before submission for publication.</p>

APPENDIX II: Published manuscripts

Filaggrin-stratified transcriptome analysis of paediatric skin identifies mechanistic pathways in atopic dermatitis.

Filaggrin-stratified transcriptomic analysis of pediatric skin identifies mechanistic pathways in patients with atopic dermatitis

Christian Cole, PhD,^a Karin Kroboth, MSc,^b Nicholas J. Schurch, PhD,^a Aileen Sandilands, PhD,^b Alexander Sherstnev, PhD,^a Grainne M. O'Regan, MRCPI,^c Rosemarie M. Watson, MD,^c W. H. Irwin McLean, DSc,^b Geoffrey J. Barton, PhD,^a Alan D. Irvine, MD,^{c,d,e} and Sara J. Brown, MD^{b,d} Dundee, United Kingdom, and Dublin, Ireland

Background: Atopic dermatitis (AD; eczema) is characterized by a widespread abnormality in cutaneous barrier function and propensity to inflammation. Filaggrin is a multifunctional protein and plays a key role in skin barrier formation. Loss-of-function mutations in the gene encoding filaggrin (*FLG*) are a highly significant risk factor for atopic disease, but the molecular mechanisms leading to dermatitis remain unclear. **Objective:** We sought to interrogate tissue-specific variations in the expressed genome in the skin of children with AD and to investigate underlying pathomechanisms in atopic skin. **Methods:** We applied single-molecule direct RNA sequencing to analyze the whole transcriptome using minimal tissue samples.

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Uninvolved skin biopsy specimens from 26 pediatric patients with AD were compared with site-matched samples from 10 nonatopic teenage control subjects. Cases and control subjects were screened for *FLG* genotype to stratify the data set. **Results:** Two thousand four hundred thirty differentially expressed genes (false discovery rate, $P < .05$) were identified, of which 211 were significantly upregulated and 490 downregulated by greater than 2-fold. Gene ontology terms for “extracellular space” and “defense response” were enriched, whereas “lipid metabolic processes” were downregulated. The subset of *FLG* wild-type cases showed dysregulation of genes involved with lipid metabolism, whereas filaggrin haploinsufficiency affected global gene expression and was characterized by a type 1 interferon-mediated stress response. **Conclusion:** These analyses demonstrate the importance of extracellular space and lipid metabolism in atopic skin pathology independent of *FLG* genotype, whereas an aberrant defense response is seen in subjects with *FLG* mutations. Genotype stratification of the large data set has facilitated functional interpretation and might guide future therapy development. (J Allergy Clin Immunol 2014;134:82-91.)

Key words: Atopic dermatitis, direct RNA sequencing, eczema, filaggrin, gene expression, single molecule, skin, tissue, transcriptome

Atopic dermatitis (AD; atopic eczema or eczema) is an itchy inflammatory skin disease with a spectrum of clinical skin phenotypes.^{1,2} The pathology of eczematous skin is characterized by epidermal intercellular edema and a barrier dysfunction resulting in increased transcutaneous water loss and increased penetration of external allergens and irritants.³ A subset of patients with AD show greater susceptibility to bacterial⁴ and viral⁵ infection.

Previous studies have investigated the transcriptome in atopic and eczematous skin by using microarray technologies and quantitative PCR, identifying a variety of pathomechanisms, including abnormalities in epidermal differentiation,⁶⁻⁸ inflammatory pathways,⁹⁻¹⁴ and lipid homeostasis.^{12,15,16} However, microarray analysis is intrinsically restricted by preselection of genes represented on an array and by annotations from which gene expression is quantified.¹⁷ Sequencing RNA is now an established methodology to study gene expression without the restriction of gene preselection.¹⁸ We chose to use single-molecule direct RNA sequencing (DRS)^{19,20} as the optimal methodology for quantification of the relatively small amounts of mRNA available from pediatric skin biopsy samples. DRS offers accurate quantification of polyadenylated RNA, avoiding bias that might

Abbreviations used

AD: Atopic dermatitis
CILP: Cartilage intermediate layer protein gene
DRS: Direct RNA sequencing
eQTL: Expression quantitative trait loci
FDR: False discovery rate
FLG: Filaggrin gene
GO: Gene ontology
STAT: Signal transducer and activator of transcription

be introduced by the steps of reverse transcription, ligation, and amplification in other forms of RNA sequencing. In addition, DRS provided information on the DNA strand from which the mRNA was transcribed; this facilitates more accurate alignment of reads to genes in the complex overlapping regions that are common in the human genome.²¹

The finding that loss-of-function mutations in the skin barrier gene encoding filaggrin (*FLG*) are strongly and significantly associated with AD risk^{22,23} has focused attention on skin barrier dysfunction as a primary pathogenic mechanism. Filaggrin deficiency appears to play a central role in the pathogenesis of AD. Filaggrin expression can be downregulated by T_H2 cytokines^{24–26} and atopic inflammation,^{27,28} whereas *FLG* intragenic copy number variation influences AD risk in a dose-dependent fashion, even in the absence of *FLG* null mutations.²⁹ Filaggrin is expressed initially as a long insoluble polypeptide, profilaggrin, which is cleaved to produce functional monomers that aggregate and align keratin filaments.³⁰ Filaggrin is thereafter degraded in a multistep proteolysis to release hygroscopic amino acids, contributing to the skin's "natural moisturizing factor."^{31,32} The mechanisms by which the multifunctional profilaggrin/filaggrin proteins lead to AD, as well as atopic asthma, allergic rhinitis, food sensitization, and peanut allergy, have been the focus of intense study over recent years.³³

We sought to undertake a comprehensive analysis of the primary molecular abnormalities in atopic skin using accurate quantification of the transcriptome in pediatric patients with AD compared with nonatopic teenage control subjects. *FLG* genotype was used to define subphenotypes for the stratification of this large data set. Uninvolved skin was chosen to study the underlying pathomechanisms of AD without the confounding effects of secondary inflammation or infection. Our strategy for analysis was stepwise, as follows: (1) all cases compared with all control subjects, (2) case-control comparison stratified by *FLG* genotype, and (3) case-case comparison stratified by *FLG* genotype (Fig 1).

METHODS

Study subjects: Patients with AD

This study was reviewed and approved by the research ethics board at Our Lady's Children's Hospital, Dublin, Ireland (reference: SAC/119/09 26). Pediatric patients with AD of Irish ethnicity attending the dermatology department were invited to participate. Twenty-six children aged 6 to 16 years and their parents/guardians provided written informed consent; 19 were male, and 7 were female (Table 1). Each child had a history of chronic relapsing AD diagnosed by experienced pediatric dermatologists (G.M.O'R., R.M.W., A.D.I., and S.J.B.) and moderate or severe disease, as defined according to the Nottingham Eczema Severity Score.³⁴ Children with a history of current or previous treatment with systemic

immunomodulatory medication were excluded. The area of biopsy was not treated with topical steroids for 4 weeks before sampling, and emollients were not applied for 2 days before biopsy. A single punch biopsy specimen of 3 to 4 mm in diameter was taken from each child after local anesthetic injection and standard aseptic technique from the upper outer buttock skin. Clinically uninvolved skin was sampled and protected skin on the buttock was chosen to minimize differences resulting from UV exposure and variation in environmental humidity.³⁵ Batch effects and RNA degradation were minimized because samples were collected by 2 clinicians (G.M.O'R. and S.J.B.), and the biopsy specimens were immediately snap-frozen in liquid nitrogen before storage in a single –80°C freezer before simultaneous processing. The study subjects had previously provided blood samples for DNA extraction as part of the ongoing National Children's Research Centre AD Case Collection.³⁶

Study subjects: Nonatopic control subjects

The collection of samples from healthy volunteers was reviewed and approved by the East of Scotland Research Ethics Service, United Kingdom (LR/11/ES/0043). Nonatopic teenage volunteers with 4 grandparents of Irish or Scottish descent were recruited. Skin biopsy specimens were collected in the same way as for the patients with pediatric AD, and 5 mL of venous blood was collected for DNA extraction.

RNA extraction and quality control

The protocol for extraction of total RNA of greater than 200 nucleotides in length from tissue by using the Qiagen RNeasy Mini Kit (Qiagen, Manchester, United Kingdom) was modified and optimized, as follows. Working on dry ice, subcutaneous fat was trimmed from the skin biopsy specimen, and the remaining sample was placed in a 2-mL sample tube RB (Qiagen, UK) with 600 μ L of Buffer RLT (Qiagen, UK) and one 7-mm stainless steel bead (Qiagen, UK). The sample was disrupted and homogenized at 4°C in a Qiagen TissueLyser LT for 5 minutes at 50 oscillations per second. RNA extraction then proceeded according to a standard protocol with 2 final elution steps each using 30 μ L of RNase-free water and centrifuged at 12,000 rpm for 90 seconds. Spectrophotometry (NanoDrop ND-1000 spectrophotometer; Thermo Scientific, Uppsala, Sweden) and a microfluidics platform for size analysis and quantification (Agilent 2100 Bioanalyser; Agilent Technologies, Santa Clara, Calif) were used for quality control. This protocol yielded 2.5 to 10.8 μ g per sample of RNA with an A_{260/280} ratio of 1.9 to 2.1.

FLG genotyping

Genomic DNA samples extracted from blood were initially screened for *FLG* null mutations with TaqMan allelic discrimination assays (TaqMan, Applied Biosystems 7700 sequence detection system; Applied Biosystems, Foster City, Calif), as previously described.³⁷ Next-generation deep sequencing of 25 cases and all 10 control subjects identified additional mutations that were confirmed by using Sanger sequencing.

Measurement of global transcript abundance

DRS was performed on a HeliScope Sequencer (Helicos, Cambridge, Mass) with 500 ng of extracted RNA, as previously described.¹⁹ RNA molecules are captured by the poly-A tail, and the single molecule of mRNA is directly sequenced from the 3' end, producing reads up to 70 bp in length (median, 32 bp).

DRS read processing

Details of sequence alignment and analysis are presented in the Methods section in this article's Online Repository at www.jacionline.org. DRS resulted in 480 million reads across all 36 samples, providing gene expression data for 11,259 genes. The raw data are deposited at the European Genome-Phenome Archive (EGAS00001000823/EGAC00001000200); processed data are available at polyAdb (<http://www.compbio.dundee.ac.uk/polyAdb>)

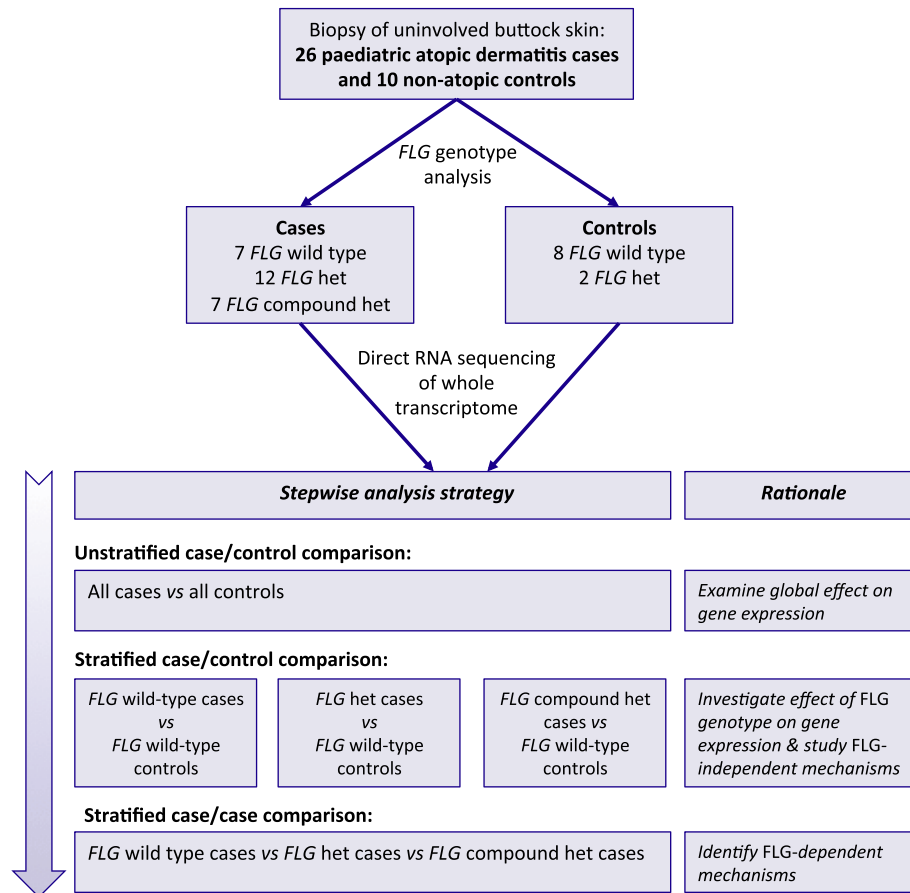


FIG 1. Flow diagram summarizing study design and analysis strategy. *het*, Heterozygous.

TABLE I. *FLG* genotype and demographic data for 26 pediatric patients with moderate-to-severe AD and 10 nonatopic control subjects

Phenotype	<i>FLG</i> genotype	No. (%)	Mean age (y [range; SD])	Male sex, no. (%)
Cases	Wild-type	7 (27)	12.3 (9-15; 2.8)	7 (100)
	Heterozygous	12 (46)	11.6 (6-16; 3.4)	8 (67)
	Compound heterozygous	7 (27)	10.7 (6-13; 3.4)	4 (57)
	Total	26	11.5 (6-16; 3.2)	19 (73)
Control subjects	Wild-type	8 (80)	17.9 (16-19; 0.8)	4 (50)
	Heterozygous	2 (20)	18.0 (18-18; 0.0)	2 (100)
	Compound heterozygous	0 (0)	NA	0 (0)
	Total	10	17.9 (16-19; 0.7)	6 (60)

Cases were diagnosed by experienced pediatric dermatologists (G.M.O'R., R.M.W., A.D.I., and S.J.B.), and severity was defined by using the Nottingham eczema severity score.³⁴ Heterozygous mutations in the cases were p.R501X (n = 6) and c.2282del4 (n = 6), and those in the control subjects were p.R501X (n = 1) and c.5690delA (n = 1). Compound heterozygous genotypes (each n = 1) were p.R501X/c.2282del4, p.R501X/p.S3247X, p.R501X/p.G1139X, p.R501X/p.S1280X, c.2282del4/p.R1474X, p.S1040X/c.10885delC, and p.S608X/p.Y2092X.
NA, Not applicable.

and can be viewed in the Integrated Genome Browser or as data tracks at www.ensembl.org. Scripts for performing analysis and generating the figures that accompany this article are available at polyAdb (<http://www.compbio.dundee.ac.uk/polyAdb>).

Differential gene expression and *FLG* correlation analyses

EdgeR (version 2.6.12 in R version 2.15.1) analysis was performed with generalized linear models to control for sex bias between comparisons. Unless otherwise stated, genes were called as significantly differentially expressed if the Benjamini-Hochberg corrected false discovery rate (FDR) was less than 0.05.³⁸ All-against-all correlation of gene expression across individual samples was performed with the Pearson method. Further details are presented in the [Methods](#) section in this article's Online Repository.

Gene ontology analysis and functional protein network analysis

Gene ontology (GO) has been developed to provide a controlled vocabulary of terms to describe the characteristics of genes and gene products with standardization across species and between databases. This allows the bioinformatic analysis of GO terms in large data sets for thematic classification. GO analyses were performed with AmiGO gene ontology version 1.8 (http://amigo.geneontology.org/cgi-bin/amigo/search.cgi?action=advanced_query). Functional protein association networks were investigated *in silico* by using STRING_{9.05} (<http://string-db.org/>).

Quantitative PCR analysis

RNA (1.4 ng) was converted to cDNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to a standard protocol. Aliquots of RNA extracted from atopic skin samples were analyzed in triplicate by using real-time quantitative PCR performed according to standard protocols with the TaqMan 7900HT Fast (Applied Biosystems) with normalization to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

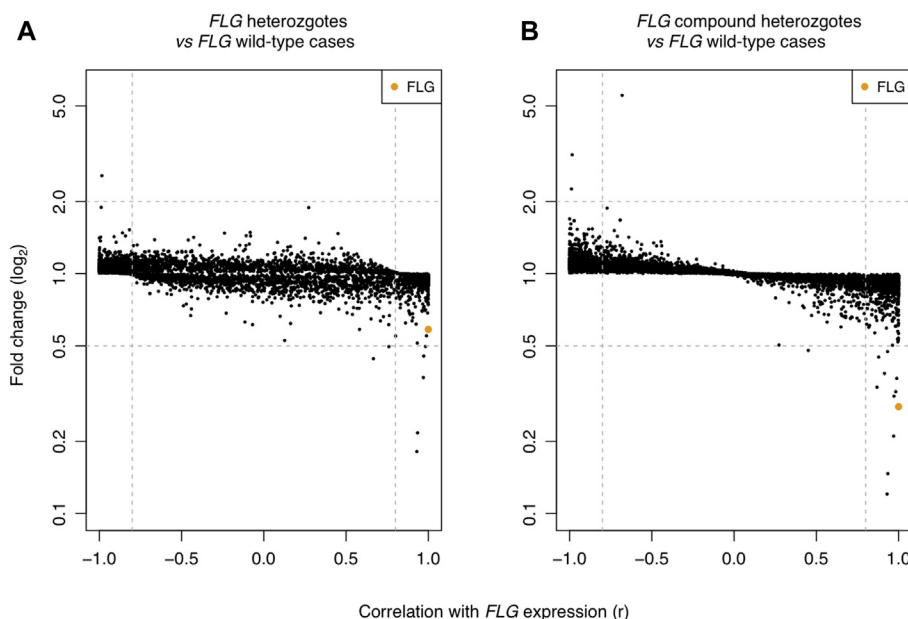


FIG 2. Scatterplots showing correlation of gene expression levels with *FLG* expression. The fold change of significantly differentially expressed genes (FDR $P < .05$) is plotted against the Pearson correlation coefficient for correlation with *FLG* expression. Each point represents expression of a single gene. *FLG* is marked in orange. **A**, Correlation of *FLG* wild-type ($n = 7$) compared with *FLG* heterozygous ($n = 12$) samples. **B**, Correlation of *FLG* wild-type ($n = 7$) versus *FLG* compound heterozygous ($n = 7$) samples.

RESULTS

Seventy-three percent of patients with AD carry 1 or more *FLG* null mutations

Our comprehensive screen for *FLG* null mutations revealed a high proportion of mutation carriers (19/26 [73%] of cases, Table I), reflecting the severity of disease and enrichment for coexisting ichthyotic skin phenotype. One heterozygous mutation, p.Y2092X, is reported for the first time. Two (20%) of 10 control subjects were heterozygous for an *FLG* null mutation (Table I).

FLG genotype affects global gene expression

Gene expression between samples was highly correlated (Pearson $r = 0.81$ – 0.99 , see Fig E1 in this article's Online Repository at www.jacionline.org), and comparison of the control samples with AD cases revealed no obvious clustering by phenotype or *FLG* genotype (see Fig E2 in this article's Online Repository at www.jacionline.org). This highlights the subtlety of changes in gene expression in uninvolved atopic skin. The samples do not cluster according to age, providing some assurance that the use of samples from older subjects in the control group does not result in bias to account for the observed differences in transcriptional profile. However, in contrast, correlation with *FLG* gene expression showed a striking global change between samples of different *FLG* genotypes (Fig 2).

"Defence response" and "extracellular region" genes are overexpressed in atopic skin, whilst "lipid metabolic processes" and "small molecule metabolic processes" are downregulated

Comparing all 26 cases with all 10 control subjects showed 2430 differentially expressed genes with an FDR of less than 0.05,

including 211 with a fold change of greater than 2.0 and 490 genes with a fold change of less than 0.5. The full list is shown in Table E1 in this article's Online Repository at www.jacionline.org. GO analysis identified the most highly significant terms in the greater than 2.0-fold upregulated genes as "defense response" (28 genes, FDR: $P = 7.1 \times 10^{-07}$), the "extracellular region" or "extracellular space" (each 32 genes, FDR: $P = 1.8 \times 10^{-3}$) and "receptor binding" (23 genes, FDR: $P = 5.7 \times 10^{-3}$). The most highly significant terms in the less than 0.5-fold downregulated genes were "lipid metabolic process" (52 genes, FDR: $P = 6.6 \times 10^{-11}$), "small molecule metabolic process" (76 genes, FDR: $P = 6.6 \times 10^{-11}$), and "organic acid metabolic process" (44 genes, FDR: $P = 6.6 \times 10^{-11}$). The full list is shown in Table E2 in this article's Online Repository at www.jacionline.org.

Analysis of the significantly upregulated genes identified predicted protein interaction networks classified as "defense response," "extracellular region," and "receptor binding" (see Fig E3 in this article's Online Repository at www.jacionline.org). Significantly downregulated genes show predicted functional networks within "lipid metabolic processes," "small molecule metabolism," and "organic acid metabolic processes" (see Fig E4 in this article's Online Repository at www.jacionline.org).

FLG wild-type subjects show dysregulation of genes involved with lipid metabolism and upregulation of extracellular matrix terms

Analysis of the 8 *FLG* wild-type control subjects compared with the 7 *FLG* wild-type cases identified 401 differentially expressed genes with an FDR of less than 0.05, including 105 with a fold change of greater than 2.0 and 87 with a fold change of less than 0.5 (the full list can be found in Table E3 in this article's Online Repository at www.jacionline.org).

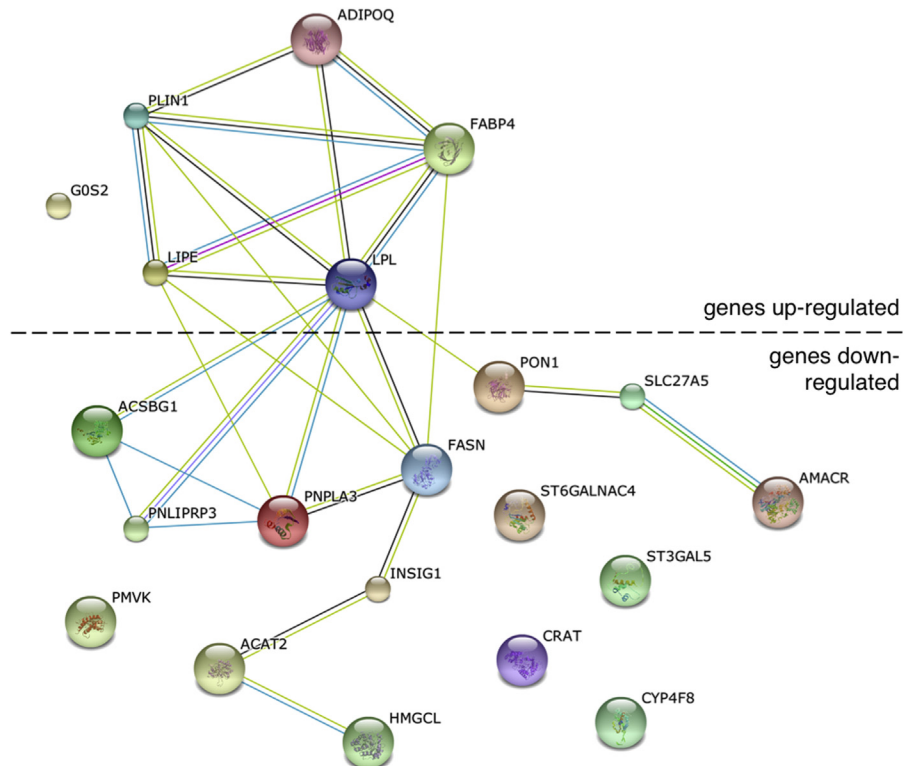


FIG 3. Network analysis of lipid metabolism genes differentially expressed in *FLG* wild-type AD cases compared with *FLG* wild-type control subjects. Twenty-one significantly differentially expressed genes (defined as FDR: $P < .05$ and fold change >2.0 or <0.05) were classified with the GO terms “lipid metabolic process” ($n = 13$), “cellular lipid metabolic process” ($n = 13$), “lipid particle” ($n = 4$), and “triglyceride catabolism” ($n = 3$). Linear connectors indicate evidence for association in published data sets (STRING_{9.05}; accessed March 3, 2014).

Significant GO terms in the transcripts upregulated more than 2.0-fold included “extracellular region” (17 genes, FDR: $P = 3.0 \times 10^{-3}$) and “lipid particle” (4 genes, FDR: $P = 3.0 \times 10^{-3}$). Significantly downregulated transcripts (fold change <0.5) were classified with the GO terms “cellular lipid metabolic process” (13 genes, FDR: $P = 1.6 \times 10^{-3}$) and “lipid metabolic process” (13 genes, FDR: $P = 2.7 \times 10^{-2}$; the full list can be found in Table E2). *In silico* protein network analysis of the differentially expressed lipid metabolism genes shows a predicted network involving both upregulated and downregulated transcripts (Fig 3).

Case-control analysis stratified by *FLG* genotype shows upregulated defense response cytokines and downregulated “steroid metabolic process”

Analysis stratified according to *FLG* genotype was performed to investigate the mechanisms by which filaggrin haploinsufficiency might predispose to the development of AD. The 8 *FLG* wild-type control subjects were compared with the 7 compound heterozygous cases, revealing 816 differentially expressed transcripts (FDR <0.05). These included 137 with a fold change of greater than 2.0 and 266 with a fold change of less than 0.5 (the full list can be found in Table E4 in this article’s Online Repository at www.jacionline.org). Significant GO terms in the upregulated transcripts included “defense response” (17 genes, FDR: $P = 2.3 \times 10^{-4}$) and “response to biotic stimulus” (14 genes, FDR: $P = 2.3 \times 10^{-4}$), “chemokine

activity” (5 genes, FDR: $P = 1.3 \times 10^{-3}$), and “chemokine receptor binding” (5 genes, FDR: $P = 1.3 \times 10^{-3}$; full list shown Table E2). *In silico* protein network analysis of the upregulated transcripts demonstrated a functional network of chemokines and cytokines in the defense response, including both T_H1 - and T_H2 -associated transcripts: *CXCL9*, *CXCL10*, *CCL13*, *CCL18*, *SELE*, *IFI27*, and *IRF1* (see Fig E5, A, in this article’s Online Repository at www.jacionline.org). The most significant GO term in the transcripts downregulated less than 0.5-fold was “steroid metabolic process” (14 genes, FDR: $P = 3.6 \times 10^{-3}$, see Table E2).

Analysis of the 8 *FLG* wild-type control subjects compared with 12 *FLG* heterozygous cases revealed 1139 differentially expressed transcripts (FDR, <0.05) in which 104 are significantly upregulated (fold change, >2.0) and 313 are significantly downregulated (fold change, <0.5 ; the full list can be found in Table E5 in this article’s Online Repository at www.jacionline.org). The upregulated transcripts showed enrichment for GO terms in the “defense response” (16 genes, FDR: $P = 7.6 \times 10^{-3}$) forming a predicted protein network analogous to that seen in the wild-type control subject versus compound heterozygous case comparison, with the addition of *GRIN2B*, *GRIK2*, and *MNDA* (see Fig E5, B). Genes classified for the ontology terms “receptor binding” (15 genes, FDR: $P = 2.9 \times 10^{-12}$), “cytokine activity” (7 genes, FDR: $P = 2.9 \times 10^{-12}$), and “extracellular region” (19 genes, FDR: $P = 3.2 \times 10^{-6}$) were also significantly upregulated. The

most highly significant GO term in the transcripts downregulated less than 0.5-fold were “small molecule metabolic process” (60 genes, FDR: $P = 3.1 \times 10^{-10}$), “carboxylic acid metabolic process,” and “oxoacid metabolic process” (each 35 genes and FDR: $P = 3.1 \times 10^{-10}$; the full list can be found in Table E2).

FLG mRNA shows a stepwise reduction in patients with AD, and there might be upregulation of expression in FLG wild-type atopic skin

FLG mRNA-normalized read counts show a stepwise reduction from FLG wild-type to FLG heterozygous and compound heterozygous patients with AD (Fig 4). This indicates that a form of nonsense-mediated decay occurs in the context of an FLG null mutation, although mature mRNA transcripts are still detectable even in those subjects with 2 FLG null mutations (Fig 4). Comparison of all cases with all control subjects showed no significant difference in FLG mRNA levels ($P > .05$), but there is significantly greater mRNA expression in wild-type AD cases than wild-type control subjects ($P = 3.0 \times 10^{-3}$), suggesting that there might be a compensatory upregulation of FLG mRNA in atopic skin of FLG wild-type subjects.

Differentially expressed transcripts at loci near to regions identified by genome-wide association studies indicate expression quantitative trait loci

Significantly differentially expressed transcripts in the case-control analysis were compared with AD-associated single nucleotide polymorphisms from published genome-wide association studies (see the Methods section in this article's Online Repository). Seventeen putative expression quantitative trait loci (*cis*-eQTLs) were identified on the basis of a transcript proximity of less than 250 kb between the single nucleotide polymorphism site and the 5' end of the transcript (see Table E6 in this article's Online Repository at www.jacionline.org).³⁹ Four of the proposed *cis*-eQTLs correspond to previously reported AD candidate genes (FLG, TNXB, C11ORF30, and ZNF652), whereas 13 represent novel candidates (see Table E6).

Analysis of cases stratified by FLG genotype shows differential expression of cartilage intermediate layer protein (CILP)

A comparison of the 26 cases stratified by FLG genotype was performed to investigate filaggrin-associated mechanisms in AD pathogenesis; this comparison also represents the most closely matched samples to control for age-specific differences. A total of 201 genes were differentially expressed (unadjusted $P < .01$); 87 genes were differentially expressed in the FLG wild-type cases versus compound heterozygous cases, and 127 were differentially expressed in the wild-type versus heterozygote comparison (see Table E7 in this article's Online Repository at www.jacionline.org). Forty-one genes showed a fold change of greater than 2.0 or less than 0.5. After controlling for multiple testing, 2 genes showed a statistically significant difference in expression: FLG (FDR: $P = 6.1 \times 10^{-12}$; fold change, 0.3) and cartilage intermediate layer protein (CILP; FDR: $P = .03$; fold change, 0.2). The differential expression of FLG, CILP, and selected other transcripts was validated by using quantitative PCR with aliquots of the previously extracted RNA samples

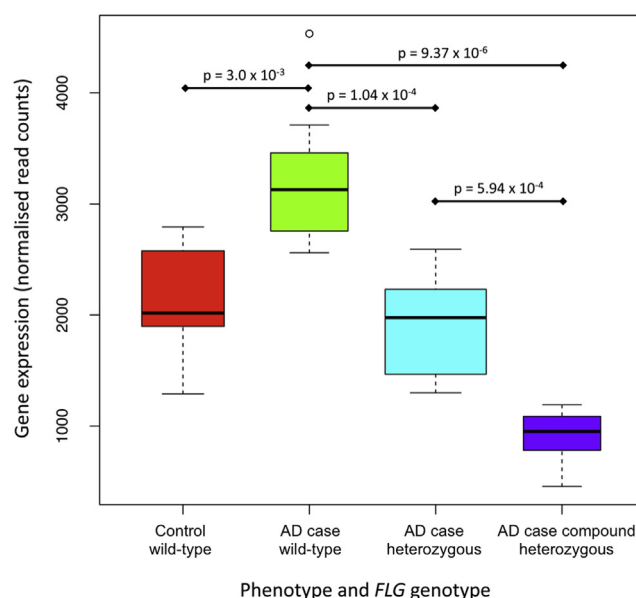


FIG 4. Box plot showing FLG mRNA read counts in different AD phenotypes and FLG genotypes. Gene expression was normalized across all samples with EdgeR: 8 wild-type control subjects, 7 wild-type AD cases, 12 heterozygous cases, and 7 compound heterozygous cases.

(see Table E8 in this article's Online Repository at www.jacionline.org).

GO analysis of the 127 transcripts showing differential expression between FLG wild-type and compound heterozygous cases showed the highest number of genes to be associated with “extracellular region” (28 genes, including FLG and CILP; FDR: $P = 8.7 \times 10^{-3}$), “carbohydrate binding” (12 genes, FDR: $P = 5.6 \times 10^{-3}$), and “calcium ion binding” (14 genes, including FLG; FDR: $P = .02$; the full list can be found in Table E9 in this article's Online Repository at www.jacionline.org).

FLG expression correlates with gene expression in the extracellular space and is anticorrelated with a network of defense response genes

Correlation of gene expression with FLG expression was used to investigate filaggrin-related mechanisms and pathways in atopic skin. Twenty genes show strong correlation with FLG expression (each $r > 0.98$ and $P < .05$), including 7 classified within the extracellular region: CA2 (carbonic anhydrase 2), COL12A1 (collagen, type XII, alpha 1), MUC1 (mucin-like 1), PIP (prolactin-induced protein), PRELP (proline/arginine-rich and leucine-rich repeat protein), SCGB1D2 (secretoglobulin, family 1D, member 2), and ZG16B (zymogen granule protein 16 homolog B; the full list can be found in Table E10 in this article's Online Repository at www.jacionline.org).

The expression levels of 6 genes were strongly anticorrelated with FLG expression (each $r < -0.98$, see Table E10); 5 of these 6 genes formed a predicted network (Fig 5). Significant GO terms associated with this network include “response to virus” (4 genes, FDR: $P = 5.1 \times 10^{-3}$), “cellular response to type I interferon,” “response to type I interferon,” and “type I interferon-mediated signaling pathway” (each 3 genes, FDR: $P = 6.3 \times 10^{-3}$). Combining these 6 anticorrelated and upregulated genes with

the 17 defense response genes that are upregulated in the *FLG* genotype-stratified case-control analyses (see Table E2) shows a common predicted functional network of “defense response” (20 genes, FDR: 5.6×10^{-18} ; Fig 6).

DISCUSSION

The identification of genes involved in the pathogenesis of AD represents a significant challenge because of the clinical heterogeneity and complexity of multiple interrelated genetic and environmental mechanisms in patients with this disease. The identification of null mutations within the gene encoding filaggrin (*FLG*) as a strong and significant risk factor for AD²² represented a fundamental breakthrough in understanding pathogenesis.³³ The strong effect of filaggrin haploinsufficiency can be used to define AD subphenotypes clinically,⁴⁰ and we have applied this insight for stratification of the large and complex data set generated by using transcriptomic analysis.

This study used DRS to quantify the whole transcriptome of atopic skin in a unique collection of pediatric AD skin biopsy specimens; it represents the largest collection of AD skin transcriptomes reported to date. Skin offers the advantage of sampling the tissue of interest, maximizing power to detect expression traits correlating with clinical phenotype.⁴¹ Importantly, the histopathology of clinically uninvolved atopic skin demonstrates an absence of inflammatory cell infiltrate, and gene expression changes are therefore likely to represent keratinocyte-related mechanisms rather than those from any other cell type. The skin of patients with AD shows epidermal barrier dysfunction, which can be demonstrated in nonlesional (clinically uninvolved) skin, as well as areas of active eczema.^{42,43} Nonlesional skin was sampled to focus on the intrinsic biological abnormality in atopic skin and to exclude, as far as possible, the secondary effects of inflammation in patients with active dermatitis and secondary infection, which would confound the mRNA profile.^{6,10} The cutaneous gene expression profile is known to vary by age, sex, and, most significantly, body site.⁴⁴ This study included case-case comparison for optimal matching in age and skin site, and our case-control samples were carefully matched for body site and sex. Age matching in the case-control analysis was limited by the availability of skin biopsy samples from healthy children; however, the data obtained from biopsy specimens of teenage volunteers showed no clustering by age (see Fig E2). Furthermore, the differentially expressed defense response genes formed an overlapping network with the age-matched case-case analyses. DRS allows accurate quantification of mRNA species. The confirmation of findings from previous microarray studies and consistency with our own quantitative PCR studies provide support for the validity of DRS as a novel technique for the investigation of AD pathogenesis.

We have shown that genes encoding proteins in the extracellular space are differentially expressed in atopic skin, with upregulation in patients with AD compared with that seen in control subjects (see Table E2). Conversely, 7 genes encoding proteins in the extracellular region are downregulated in strong correlation with *FLG* expression (see Table E10). These genes are likely to contribute to mechanisms by which a quantitative reduction in intracellular filaggrin levels results in the paracellular barrier defect that is observed *in vitro*.^{45,46} Expression of *CILP* shows the most significantly reduced expression in *FLG* null cases compared with *FLG* wild-type cases and might represent a novel AD candidate gene. The protein encoded by *CILP* is expressed in many tissues,

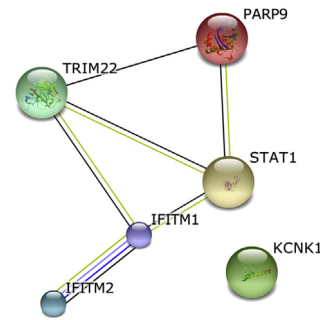


FIG 5. Network analysis of 6 genes with expression strongly anticorrelated with *FLG* expression: *TRIM22* (tripartite motif containing 22), *KCNK1* (potassium channel, subfamily K, member 1), *PARP9* (poly (ADP-ribose) polymerase family, member 9), *IFITM2* (interferon induced transmembrane protein 2), *IFITM1* (interferon induced transmembrane protein 1), and *STAT1* (STRING_{9.05}; accessed October 27, 2013).

including skin and blood, as well as articular cartilage. It is secreted into the extracellular space and sequesters growth factors, cytokines, and matrix metalloproteases in the extracellular matrix. Also, it has been shown to antagonize the actions of TGF- β 1 and insulin-like growth factor 1.^{47,48} We hypothesize that a reduction in expression of the cartilage intermediate layer protein permits increased activity of insulin-like growth factor 1 and TGF- β 1, leading to cellular proliferation, whereas a reduction in the sequestration of proinflammatory cytokines and metalloproteases in the extracellular space might simultaneously contribute to skin barrier dysfunction in cases of AD associated with filaggrin deficiency.

The second major finding of this global transcriptomic analysis is the dysregulation of lipid metabolic pathways both in the unstratified case-control comparison (see Fig E4, A) and in the *FLG* wild-type case-control comparison (Fig 3). The demonstration of lipid dysregulation predominantly in the cases without *FLG* mutations is in keeping with *in vivo*⁴⁹ and *in vitro*⁵⁰ findings that filaggrin deficiency does not affect lipid composition in the stratum corneum. Previous microarray analyses have shown a reduction in expression of lipid homeostatic genes¹² and reduced intercellular lipid levels.¹⁵ Organotypic culture of primary keratinocytes has shown increased expression of a cluster of genes associated with lipid metabolism throughout differentiation in parallel with increasing barrier properties.⁵¹ Lipid raft disruption produces transcriptomic changes in cultured keratinocytes, including disruption of cholesterol biosynthesis, that mimic changes seen in patients with AD.¹⁶ The predicted functional network in Fig 3 comprises upregulated and downregulated lipid metabolism genes and might offer insight into the complex interplay of metabolic dysfunction with systemic inflammation.⁵² Genes encoding proteins involved with very long-chain fatty acid CoA ligase activity are downregulated, which is in keeping with the observation that ceramides and long-chain fatty acids play an important role in skin barrier formation.^{49,53,54} Our stratified analysis indicates that therapies aimed to restore skin lipid composition might be most beneficial to *FLG* wild-type patients.

The *FLG*-stratified case-control and case-case comparisons have identified an overlapping functional network of proteins forming a type 1 interferon-mediated defense response (Fig 6). The upregulation of this network might relate to the dysfunctional cutaneous response to viral infection in patients with AD, which

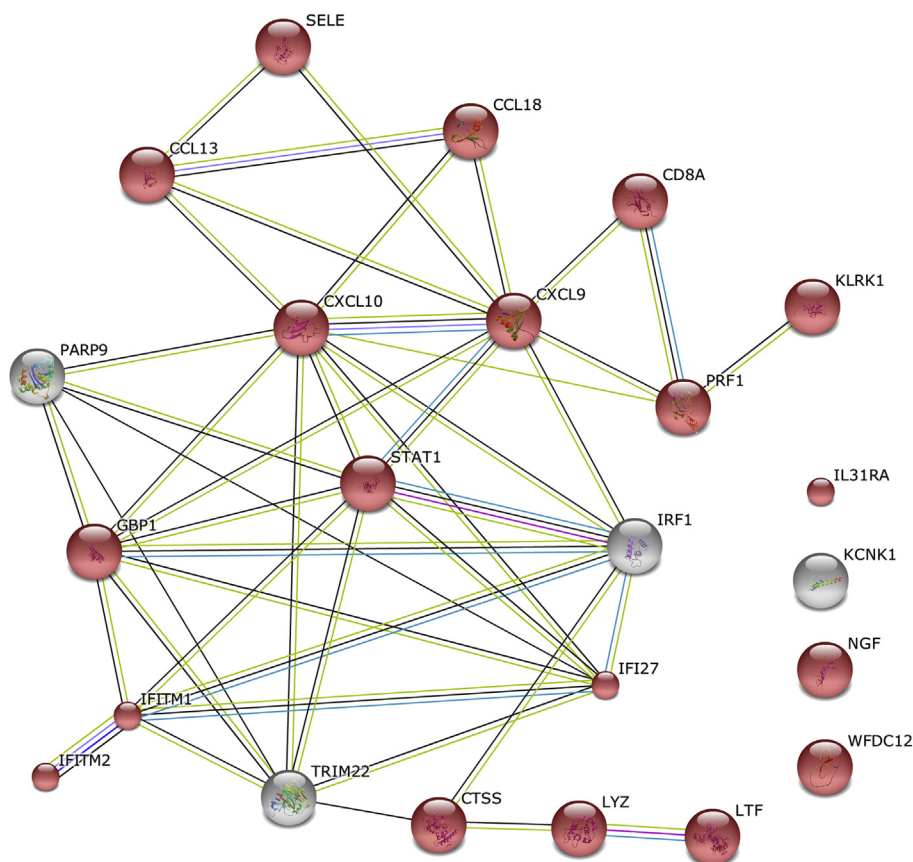


FIG 6. Genes anticorrelated with *FLG* and upregulated in *FLG* mutant cases show a common network of stress response. Six genes are anticorrelated (upregulated) with *FLG* expression, and 17 genes are upregulated in the *FLG* genotype-stratified case-control analyses. Proteins encoded by genes classified with the GO term “response to stress” are colored red (STRING_{9.05}; accessed November 16, 2013).

can be a significant problem in clinical practice.⁵ Alternatively, it might represent a suboptimal, partially functional mechanism to compensate for the increased frequency of viral infections, including eczema herpeticum,⁵⁵ seen in filaggrin-deficient subjects. The signal transducer and activator of transcription (STAT) encoded by *STAT1* contributes to the transcriptional control of several interferon-stimulated genes, including *IFITM1*, *IFITM2*, *IFI27*, and *GBP1*. The Janus kinase/STAT signaling pathway plays a key role in transmembrane signaling from the T_H2 cytokines IL-4 and IL-13, which predominate in acute AD.⁵⁶ *IFITM1* and *IFITM2* encode interferon-induced transmembrane proteins, which contribute to the control of cell growth through a multimeric complex involved in the transduction of antiproliferative and homotypic adhesion signals; they are induced by IFN- γ in primary keratinocytes *in vitro* and have been proposed to play a role in keratinocyte apoptosis in patients with AD.⁵⁷ The chemokine network encoded by genes including *CCL13*, *CCL18*, *CXCL9*, and *CXCL10* has been implicated in the pathogenesis of AD in some of the previous microarray studies.^{9,11,13,58} Filaggrin haploinsufficiency increases the risk of eczema herpeticum,⁵⁹ and the functional network predicted by transcriptomic analysis indicates a pathway that might be targeted for therapeutic intervention in susceptible patients.

The quantitative reduction in filaggrin mRNA with *FLG* null mutations (Fig 4) is consistent with previous studies showing a stepwise reduction in filaggrin breakdown products

in *FLG* heterozygotes and *FLG* homozygotes or compound heterozygotes.^{27,60} One previous microarray study has used *FLG* genotype-stratified analysis⁸ and reported no significant difference in *FLG* mRNA levels between *FLG* wild-type cases and control subjects. In our analysis comparison of all cases with all control subjects also showed no difference in *FLG* mRNA counts ($P > .05$), but we have shown a significant difference between *FLG* expression in wild-type control subjects compared with wild-type cases (Fig 4). This is compatible with the confidence range of data published previously⁸ but suggests that there might be compensatory upregulation of *FLG* expression in our pediatric AD cases. An alternative explanation is that there might be lower filaggrin expression in our control subjects, possibly reflecting the slightly older age, but detailed studies of filaggrin expression changes with aging are not currently available.

Intercellular edema (spongiosis) is a characteristic feature in patients with AD, but the underlying mechanisms are unclear. The differential expression of genes encoding proapoptotic and antiapoptotic proteins (including *IGFBP6*, *CLU*, *IFITM1*, *IFITM2*, *SELE*, *CXCL10*, *PRF1*, and *IRF1*) might contribute to the propensity to keratinocyte apoptosis that some authors consider to be a key pathomechanism in atopic spongiosis.^{57,61,62} Alternatively, the dysregulation of proteins in cell death pathways might reflect the specialized process of keratinocyte cell death, cornification.^{63,64}

The majority of AD risk loci identified by using genome-wide association studies are located within intergenic regions of unknown function. Our analysis has offered insight into possible genetic mechanisms associated with 14 of the previously reported AD risk loci. We propose *cis*-eQTLs indicating a range of pathomechanisms, including structural (*FLG*, *LCE3E*, and *TNXB*), immune response (*CST6*, *HLA-DRA*, *IRF1*, and *PRRT1*), transcriptional regulation (*ATF6B*, *C11orf30*, *RP11-21L23.4*, *RPL3P2*, *RPSAP47*, *SIPA1*, and *ZNF652*), mitochondrial (*TST*), and lipid biosynthetic (*AGPAT1*).

The use of DRS has allowed detailed study of tissue-specific gene expression data from small amounts of tissue. This transcriptomic analysis has provided new insight into the mechanistic pathways in atopic skin, which are both dependent and independent of *FLG* genotype. The strength of using a functional genotype for phenotype stratification is apparent, and this approach might prove useful for other tissue-specific inflammatory disorders and personalized medicine in the future.

We thank the children with AD who provided skin biopsy samples for this study, and we also thank the Tayside Tissue Bank, Dundee, United Kingdom, for secure sample storage of skin tissue from the cases and control subjects. Finally, we thank Mrs Linda Campbell for her expert assistance in DNA extraction and *FLG* genotyping.

Key messages

- Atopic skin shows differential gene expression in pathways classified in the extracellular space, lipid metabolism, and stress response.
- Substratification of the whole transcriptome data set according to *FLG* genotype reveals a type 1 interferon-mediated stress response in filaggrin-deficient skin.
- These findings offer insight into the underlying abnormalities in uninfamed atopic skin and might guide future therapy development.

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Filaggrin genotype does not determine the skin's threshold to UV-induced erythema.

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Filaggrin genotype does not determine the skin's threshold to UV-induced erythema



To the Editor:

Profilaggrin and filaggrin play multiple roles in the formation and function of the epidermal barrier, contributing to protection against dehydration, mechanical stress, infection, and, it has been proposed, photodamage.¹ Loss-of-function mutations in the gene encoding filaggrin (*FLG*) represent the strongest and most significant genetic risk factor for atopic dermatitis (AD) identified to date.¹ Proteolysis of filaggrin releases histidine and other amino acids into the stratum corneum. Histidine is converted by the enzyme histidase (histidine ammonia-lyase) to *trans*-urocanic acid (*trans*-UCA), which can then undergo photoisomerization on absorption of UVB to produce *cis*-UCA (see Fig E1 in this article's Online Repository at www.jacionline.org). There is experimental evidence to suggest that *cis*-UCA has immunomodulatory and photoprotective effects.

TABLE I. Demographic data and *FLG* genotype results for 71 volunteers with clinically normal skin

Sex	43 male/28 female
Age (y), range (median)	22-70 (41)
<i>FLG</i> wild-type subjects (no.)	61
<i>FLG</i> heterozygotes (no.)	10
Total (no.)	71

Volunteers were screened for the 6 most prevalent *FLG* loss-of-function mutations in the population. Five subjects were heterozygous for R501X, 3 were heterozygous for 2282del4, 1 was heterozygous for R2447X, and 1 was heterozygous for S3247X. No 3673delC or 3702delG mutations were detected, and there were no homozygotes or compound heterozygotes. Fitzpatrick skin phototype was recorded for 45 of 71 subjects, and there was no significant difference ($P = .14$, χ^2 test) in skin phototypes between the genotype subgroups.

The local and systemic immunosuppressive effects of *cis*-UCA were initially demonstrated in murine models, and more recently, histidinemic mice deficient in cutaneous UCA because of a mutation in *Hal*, the gene encoding histidase, have been reported to show increased propensity to UVB-induced DNA damage.² Mice deficient in caspase-14 (an enzyme in the profilaggrin-filaggrin proteolytic pathway) show accumulation of cyclobutane pyrimidine dimers in response to UVB radiation and increased apoptosis in the epidermis, indicating a role for caspase-14 in UVB scavenging within the stratum corneum.³ The immunosuppressive effects of *cis*-UCA have been demonstrated in human keratinocytes and leukocytes *in vitro*; knockdown of *FLG* in organotypic culture results in increased susceptibility of keratinocytes to UV-induced apoptosis.⁴ Loss-of-function mutations and copy number variation in *FLG* are known to result in lower levels of filaggrin breakdown products, including UCA, in human stratum corneum. Therefore it has been postulated that *FLG* genotype might in part determine the photoprotective capacity of human skin (see Fig E1),¹ but experimental evidence *in vivo* is lacking.

We aimed to test the hypothesis that filaggrin deficiency resulting from loss-of-function mutations in *FLG* is associated with increased erythema sensitivity to UV radiation. Cutaneous response to UV radiation was assessed by using the minimal erythema dose (MED; the lowest dose of UV causing just perceptible skin redness) as a quantifiable surrogate end point for cutaneous damage. We used detailed monochromator phototesting of 71 adult volunteers of white European ethnicity with clinically normal skin; the demographic characteristics are summarized in Table I. A calculation performed before this study commenced indicated that 7 or 8 *FLG* mutation carriers within a total study size of 70 to 80 subjects would provide sufficient statistical power to detect a 1.8-fold difference in MED. This sample size estimation was based on known variability in MEDs from previous studies and assuming comparisons of arithmetic means of log-transformed data (therefore able to back-transform differences into fold differences). Details of the power calculation are shown in the Methods section in this article's Online Repository at www.jacionline.org.

This work was approved by the East of Scotland Research Ethics Committee (reference 14/ES/0030), and the study was conducted in accordance with the Declaration of Helsinki.

Participants were screened for the 6 most prevalent loss-of-function mutations in *FLG* in the white European population (R501X, 2282del4, R2447X, S3247X, 3673delC, and 3702delG) by using published methodology.⁵ Ten (14%) of 71

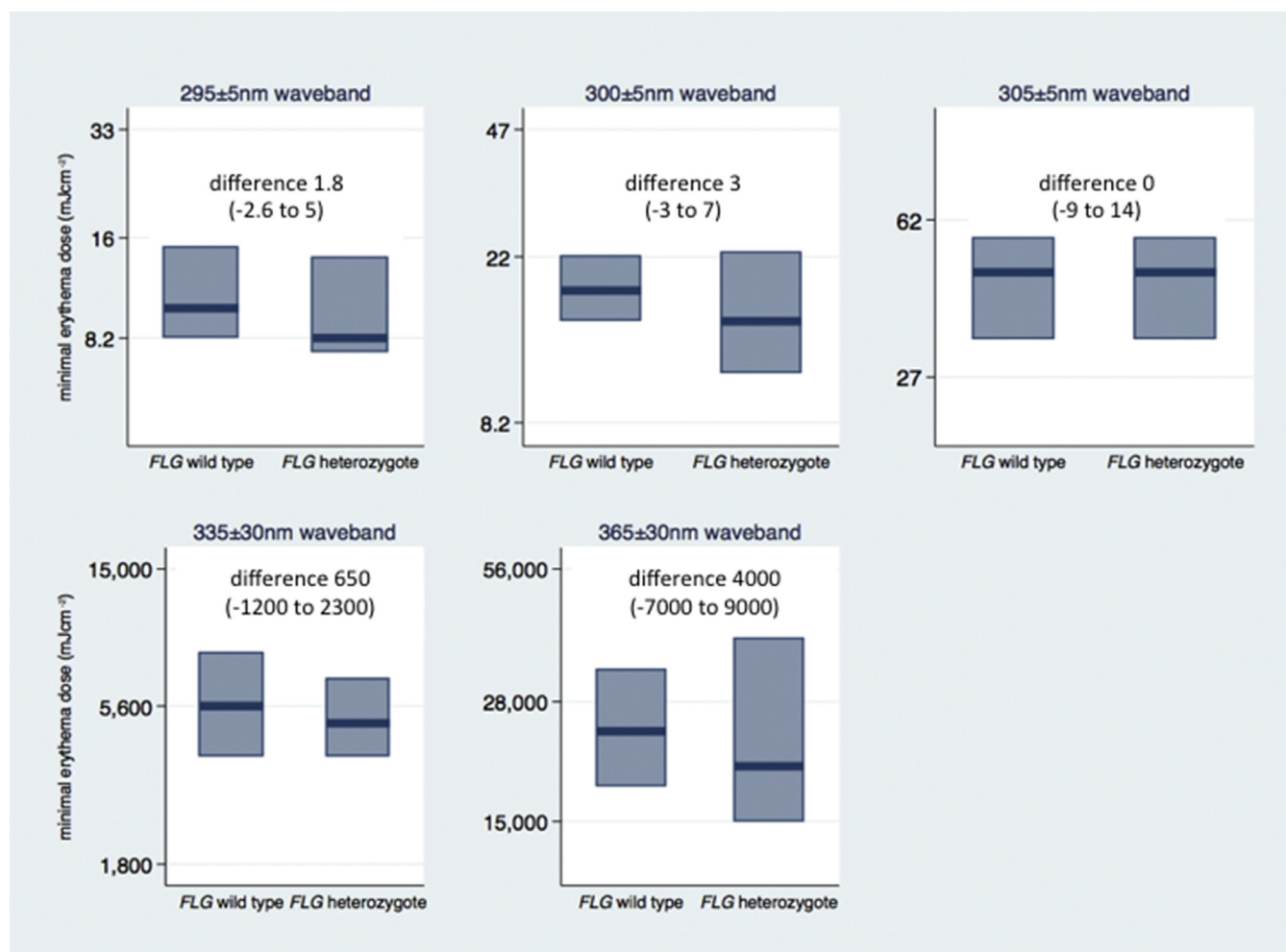


FIG 1. Box plots showing monochromator phototesting MED results in healthy volunteers of different *FLG* genotypes. The findings for 5 distinct wavebands are shown. Results for the 400 ± 30 and 430 ± 30 nm wavebands showed no difference between *FLG* wild-type and heterozygotes (see Table E1); these data are not displayed because the median MEDs and ranges are not quantifiable. Boxes indicate interquartile ranges, and the bar within each box marks the median result. The difference in median MEDs (and 95% CIs) are shown above each plot. All values are in millijoules per square centimeter. Median MEDs were compared by using the Mann-Whitney *U* test. There were 61 *FLG* wild-type subjects and 10 *FLG* heterozygous subjects tested in each group, with the exception of the 295 nm and 300 nm wavebands, in which data were obtained on 53 *FLG* wild-type subjects and 8 *FLG* heterozygous subjects.

were found to be heterozygous for a loss-of-function mutation in *FLG* (Table 1). Fitzpatrick sun-reactive skin phototype was recorded for 45 of 71 subjects, and no difference was detected ($P = .14$, χ^2 test) in skin phototypes between the genotype subgroups.

Up to 7 separate wavebands from 295 to 430 nm, representing a spectrum from UVB to UVA and visible light, were tested on the 71 subjects. A detailed description of phototesting methods is given in the Methods section in this article's Online Repository. Subjects were grouped according to *FLG* genotype, and MEDs were compared by using nonparametric rank-based methods (because some MED values were greater than or less than test dose ranges) with the Mann-Whitney *U* test (see the Methods section in this article's Online Repository) to derive CIs for differences in median MEDs (see Table E1 in this article's Online Repository at www.jacionline.org). We detected no significant differences in MEDs (defined as $P \leq .05$) between the *FLG*

wild-type and *FLG* heterozygous groups at any of the wavebands tested (Fig 1 and see Table E1). The CIs for differences were sufficiently narrow to make any large differences in MEDs between the genotype groups unlikely.

It has previously been reported that AD might be associated with photosensitivity,⁶ a lower threshold to UVB-induced erythema,⁷ or both. Some epidemiologic data also suggest a higher incidence of multiple nonmelanoma skin cancers in subjects with a history of AD.⁸ Loss-of-function mutations in *FLG* are strongly associated with AD, and there is widespread downregulation of filaggrin expression in the skin of patients with atopic eczema, which has been demonstrated at the transcriptome level by means of direct RNA sequencing,⁹ and in the breakdown products of filaggrin in the stratum corneum, which was quantified by means of HPLC.¹⁰ A partial reduction in expression of filaggrin might result from the effect of circulating inflammatory cytokines, whereas a more profound deficiency results from

loss-of-function mutations in *FLG* leading to near-complete absence of profilaggrin in the homozygous or compound heterozygous state. Therefore it can be hypothesized that filaggrin deficiency contributes to the observed photosensitivity and/or reduced threshold to UVB-induced erythema in patients with AD. We have performed a detailed analysis of cutaneous photoresponse in clinically normal skin to avoid the confounding effects of atopic inflammation. Our findings have excluded a large effect of *FLG* genotype on photosensitivity (≥ 1.8 -fold difference in MED) at any of the wavebands tested. In addition, the results of our monochromator phototesting did not indicate a differential erythema sensitivity within the wavelengths representing UVB, as would be predicted from the known absorption spectrum of UCA.

One limitation of our study is that the healthy volunteers did not include any subjects with ichthyosis vulgaris, and therefore we have not excluded the possibility that *FLG* homozygous (or compound heterozygous) subjects might show greater erythema sensitivity than wild-type subjects. However, *FLG*-null heterozygosity has a significant effect on filaggrin expression *in vivo*,^{9,10} and therefore we would expect an effect to be observed in *FLG* heterozygotes if this was substantial.

The fact that observations of UVB-induced damage in murine and *in vitro* models have not been supported by clinical data suggest that different mechanisms lead to cutaneous erythema *in vivo* than the markers of UV damage studied *in vitro* and in mice. For example, apoptosis is known to occur within areas of skin damaged by UV exposure, and this is associated with cutaneous erythema, but the relationship is nonlinear. Furthermore, the photoprotective effect of the *FLG* wild-type genotype might be attributable to a mechanical filtering of UV radiation by the stratum corneum rather than by chemical photoimmunosuppression.

In conclusion, our *FLG* genotype-stratified analysis of responses to UV and visible radiation in clinically normal skin does not support the hypothesis that the breakdown products of filaggrin play a major role in the sensitivity of human skin to UV-induced erythema. This has relevance to the ongoing search for predictors of patient response in phototherapy for AD and for the development of personalized medicine.

We thank the patients and volunteers who participated in this study and Lynn Fullerton, who provided technical support in the photobiology investigations. We are very grateful to Professors James Ferguson and Peter Farr for their expert advice in the design and conduct of these studies.

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Epidermal thymic stromal lymphopoietin predicts the development of atopic dermatitis during infancy



To the Editor:

To establish a primary prevention strategy for atopic dermatitis (AD), it is important to identify biomarkers that can predict the occurrence of AD. This study aimed to evaluate the expression level of epidermal proteins by using a tape stripping method to determine whether these proteins can be used as biomarkers predictive of AD development in infants.

In this prospective birth cohort study, we followed 75 infants in a risk group and 12 in a control group for 2 years (Fig 1). The control group consisted of infants with both parents who had neither allergy nor immediate skin test reactivity to 8 common inhalant allergens (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, tree pollen mixture I & II, weed pollen mixture, grass pollen mixture, cat, and cockroach). The risk group was defined

METHODS

Assessment of MEDs of different wavebands in healthy volunteers

Healthy adults with clinically normal skin of phototypes I to III^{E1} who had previously participated in research studies within the National Photobiology Unit (Dundee, Scotland, United Kingdom) were invited to participate in this study. Exclusion criteria were history of skin allergy, eczema/AD, psoriasis or polymorphic light eruption, immunosuppression caused by medications or disease, photosensitizing medication, and a holiday abroad or sunbed use within the preceding 4 weeks. A total of 71 subjects provided written informed consent and a saliva sample for DNA extraction. Emollient application to skin test sites was not allowed within the 48 hours before phototesting.

Detailed phototesting was undertaken by using an irradiation monochromator, which is a diffraction grating device with a 1.6-kW or 450-W xenon arc lamp.^{E2,E3} This instrument allows irradiation of small areas of the skin over a range of wavebands, which are included in the solar spectrum (UVB to UVA/visible). Monochromator phototesting was performed as follows. On day 1, approximately 1-cm² areas of the skin on the volunteer's back were exposed to UV and visible light according to a standardized procedure established in the National Photobiology Unit. A range of doses of UV and visible light at specific narrow wavebands was used for all subjects centered on (with half-maximum bandwidth) 295 ± 5, 300 ± 5, 305 ± 5, 335 ± 30, 365 ± 30, 400 ± 30, and 430 ± 30 nm.

The MED, which was defined as the minimum dose producing just perceptible erythema for each waveband tested, was determined 24 hours after irradiation. The irradiation procedure was repeated on day 2 on a separate area of back skin using smaller dose increments (10% to 20%) across a narrower range of doses at each waveband selected on the basis of the MEDs seen 24 hours after first irradiation to establish the MED precisely. Final MEDs were assessed 24 hours later (day 3).

Power calculation

Our prestudy sample size calculation was performed to determine how many subjects were likely to be needed to detect a clinically important difference in MEDs between the *FLG* genotype groups within the 305 ± 30 nm waveband. MED data derived from testing with a geometric dose series do not follow a normal distribution, and therefore we based our sample size on the minimum difference in arithmetic means of natural log-transformed MEDs. This method was used because differences in arithmetic means of log-transformed data can be "back-transformed" to fold differences (eg, 1.8-fold), which is more understandable than the difference in log-transformed MEDs that equates to this.

MEDs for the 305 ± 5 nm waveband (representing a narrow waveband in the UVB region, the waveband of interest) from 120 healthy volunteers tested at the National Photobiology Unit were used to derive variance. These nonnormal data were log-transformed, and the numbers needed to detect a 1.5-, 1.8-, and 2-fold difference in geometric means were estimated by using Stata 12.1 software (StataCorp, College Station, Tex). Assuming that approximately 10% of the study participants would have 1 or more *FLG* loss-of-function mutations,^{E4} to obtain an 80% power with a *P* value of .05, we required at least 7 or 8 subjects with *FLG* mutations (expected within a total sample size of 70-80 subjects) to detect a difference of 1.8-fold in mean natural log-transformed MEDs at this waveband between the wild-type and *FLG*-null groups. Similarly, 55 volunteers, including 5 or 6 *FLG* mutation carriers, were needed to detect a 2-fold difference in mean natural log-transformed UVB MEDs.

Therefore the results arising from our sample size, including 71 volunteers and 10 *FLG* mutation carriers, have sufficient statistical power to effectively exclude an association with *FLG* genotype and erythema response of a 1.8-fold or greater difference at the wavebands tested.

Statistical analysis of monochromator phototesting data

When analyzing our data, we used nonparametric methods reliant on ranks, rather than absolute values, instead of parametric methods based on transformed data. This was for the practical reason that although we obtained ranks

for all MEDs, the precise values for some were unknown (greater than or less than our test dose range). Some MEDs were determined to be greater than or less than the test irradiation ranges. A small number was added to all MEDs at greater than the top dose tested for wavebands of 305 nm and longer and a small number was subtracted from MEDs of less than the lowest dose tested to allow appropriate rank-based analyses. The phototesting results were compared with *FLG* genotype status (*FLG* wild-type or *FLG* mutant) by using the Mann-Whitney *U* test to analyze these nonparametric data to test the null hypothesis that there was no association of MEDs with genotype. Corresponding CIs around medians were derived by using the methods of Altman and Gardner.^{E5} We took a *P* value of .05 or less to be significant.

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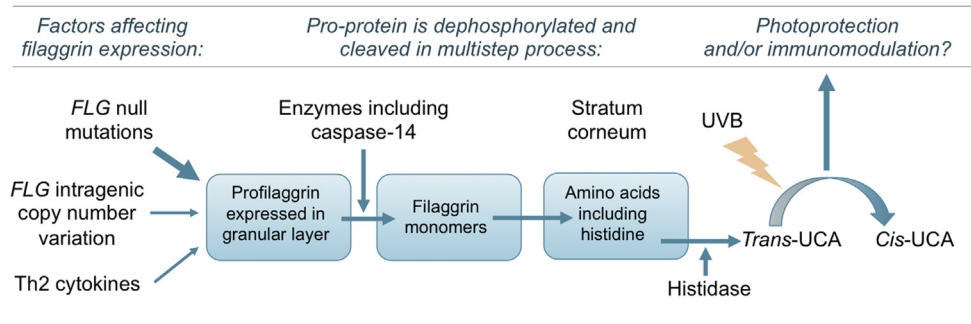


FIG E1. Diagrammatic summary of factors affecting the profilaggrin-*cis*-UCA pathway. Previous studies have demonstrated the effects of variation within *FLG*^{E6-E8} and levels of T_H2 cytokines^{E9,E10} on filaggrin expression. The role of caspase-14 in profilaggrin processing has been illustrated in mice.^{E11} Filaggrin is degraded to release a pool of amino acids rich in histidine^{E12} in the stratum corneum, contributing to barrier function through hydration and acidification.^{E13} The conversion of histidine to *trans*-UCA is catalyzed by histidase,^{E14} and the *trans*-isomer is converted to *cis*-UCA by UVB.^{E15} UVB absorption might contribute to cutaneous photoprotection, and *cis*-UCA might have additional immunomodulatory effects.^{E16-E20}

TABLE E1. Results of monochromator phototesting of 71 healthy volunteers stratified according to *FLG* genotype

	295 ± 5 nm	300 ± 5 nm	305 ± 5 nm	335 ± 30 nm	365 ± 30 nm	400 ± 30 nm	430 ± 30 nm
Waveband	Median MED (mJ/cm ²)						
<i>FLG</i> wild-type subjects	10.0 (n = 53)	18.0 (n = 53)	47.0 (n = 61)	5,600 (n = 61)	24,000 (n = 61)	>82,000 (n = 61)	>82,000 (n = 61)
<i>FLG</i> heterozygous subjects	8.2 (n = 8)	15.0 (n = 8)	47.0 (n = 10)	4,950 (n = 10)	20,000 (n = 10)	82,000 (n = 10)	>82,000 (n = 10)
Difference	1.8 mJ/cm ² higher in <i>FLG</i> wild-type	3.0 mJ/cm ² higher in <i>FLG</i> wild-type	No difference	650 mJ/cm ² higher in <i>FLG</i> wild-type	4,000 mJ/cm ² higher in <i>FLG</i> wild-type	Not quantifiable	Not quantifiable
95% CI for difference	−2.6 to 5.0	−3.0 to 7.0	−9.0 to 14.0	−1,200 to 2,300	−7,000 to 9,000	Not quantifiable	Not quantifiable
<i>P</i> value (Mann-Whitney <i>U</i> test)	.41	.35	.79	.62	.74	.58	.70

Some cells are “not quantifiable” because results of greater than the highest test dose were recorded.

Functional and proteomic analysis of a full thickness filaggrin-deficient skin organoid model.



RESEARCH ARTICLE

REVIEWED Functional and proteomic analysis of a full thickness filaggrin-deficient skin organoid model [version 2; peer review: 3 approved]

Previously titled: Proteomic analysis of a filaggrin-deficient skin organoid model shows evidence of increased transcriptional-translational activity, keratinocyte-immune crosstalk and disordered axon guidance

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Abstract

Background: Atopic eczema is an itchy inflammatory disorder characterised by skin barrier dysfunction. Loss-of-function mutations in the gene encoding filaggrin (*FLG*) are a major risk factor, but the mechanisms by which filaggrin haploinsufficiency leads to atopic inflammation remain incompletely understood. Skin as an organ that can be modelled using primary cells *in vitro* provides the opportunity for selected genetic effects to be investigated in detail.

Methods: Primary human keratinocytes and donor-matched primary fibroblasts from healthy individuals were used to create skin organoid models with and without siRNA-mediated knockdown of *FLG*. Biological replicate sets of organoids were assessed using histological, functional and biochemical measurements.

Results: *FLG* knockdown leads to subtle changes in histology and ultrastructure including a reduction in thickness of the stratum corneum and smaller, less numerous keratohyalin granules. Immature organoids showed some limited evidence of barrier impairment with *FLG* knockdown, but the mature organoids showed no difference in transepidermal water loss, water content or dye penetration. There was no difference in epidermal ceramide content. Mass spectrometry proteomic analysis detected >8000 proteins per sample. Gene ontology and pathway analyses identified an increase in transcriptional and translational activity but a reduction in proteins contributing to terminal differentiation, including caspase 14, dermokine, AKT1 and TGF-beta-1. Aspects of innate and adaptive immunity were

Open Peer Review

Reviewer Status

	Invited Reviewers		
	1	2	3
version 2 published 26 Nov 2019	 report		
version 1 published 09 Sep 2019	 report	 report	 report

- Sabine Werner**, ETH Zurich (Swiss Federal Institute of Technology Zürich), Zürich, Switzerland
- Ulrich auf dem Keller**, Technical University of Denmark, Kongens Lyngby, Denmark
- Neil Rajan** , Newcastle University, Newcastle upon Tyne, UK
- Sanja Kezic**, University of Amsterdam, Amsterdam, The Netherlands

represented in both the up-regulated and down-regulated protein groups, as was the term 'axon guidance'.

Conclusions: This work provides further evidence for keratinocyte-specific mechanisms contributing to immune and neurological, as well as structural, aspects of skin barrier dysfunction. Individuals with filaggrin deficiency may derive benefit from future therapies targeting keratinocyte-immune crosstalk and neurogenic pruritus.

Keywords

Axon guidance, atopic dermatitis, eczema, filaggrin, gene ontology, keratinocyte-immune crosstalk, organoid, proteomics

Any reports and responses or comments on the article can be found at the end of the article.

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Author roles: **Elias MS:** Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Wright SC:** Data Curation, Formal Analysis, Investigation, Methodology, Validation, Visualization, Writing – Review & Editing; **Nicholson WV:** Formal Analysis, Methodology, Visualization, Writing – Review & Editing; **Morrison KD:** Formal Analysis, Methodology, Validation, Visualization, Writing – Review & Editing; **Prescott AR:** Data Curation, Investigation, Methodology, Resources, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Ten Have S:** Data Curation, Formal Analysis, Investigation, Methodology, Resources, Software, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Whitfield PD:** Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Resources, Supervision, Writing – Original Draft Preparation, Writing – Review & Editing; **Lamond AI:** Funding Acquisition, Methodology, Project Administration, Resources, Supervision, Writing – Review & Editing; **Brown SJ:** Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Resources, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

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The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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REVISED Amendments from Version 1

The main aim of this publication is to facilitate sharing of the global mass spectrometry data generated from the skin organoid models with *FLG* knockdown (described in detail) and functional analyses (described and reported); this main aim has been clarified.

In response to the interest from Reviewers 1 and 3, we have provided an explanation as to why knockdown of *FLG* at mRNA level is demonstrated to a lesser degree than the knockdown at protein level, reflecting the prolonged duration (10 days) of organoid culture.

We have emphasised that increased dye penetration was observed in some but not all of the immature organoids, as requested by Reviewer 1.

Additional details have been provided to explain how the protein false discovery rate was determined for the removal of false positives, as requested by Reviewer 1.

We have included a fuller explanation of the analysis approach, in which we focus on consistent differences observed across the biological replicate experiments and use thresholds for increased and decreased expression to include roughly equal numbers of proteins.

We have corrected the use of the terms 'ratio' and 'fold-change' where necessary.

A new Figure 10 is provided, following advice from reviewer 2. The colour-coding has been removed and we have labelled selected data points. During revision of this figure we discovered an error in the plotting of this figure; this detail has been corrected in the revised Methods section and in the re-drawn volcano plot.

The title has been modified to include the term 'full thickness', to clarify the complexity of our model (as recommended by reviewer 2). The list of main findings has now been removed from the title because it had become too long.

Minor typos have been corrected, including two references numbered incorrectly in the previous manuscript.

Any further responses from the reviewers can be found at the end of the article

Introduction

Atopic eczema (also termed atopic dermatitis or eczema^{1,2}) is an itchy inflammatory skin disorder with complex and multifactorial aetiology³. The phenotype is heterogeneous and pathogenic mechanisms vary between affected individuals⁴. The pathogenesis may include differential contributions of: immune activation and skin barrier dysfunction; local and systemic effects; multiple genetic and environmental factors⁵. Loss-of-function mutations in *FLG*, encoding filaggrin, cause the common Mendelian disorder of keratinization, ichthyosis vulgaris (OMIM # 146700). *FLG* null mutations are also strongly associated with increased risk of atopic eczema^{6,7} and multiple other atopic traits^{8–10}.

Skin is an organ that can be modelled *in vitro* to effectively recapitulate the multi-layered structure and gene expression patterns of human skin *in vivo*¹¹. This offers the opportunity for selected molecular mechanisms to be investigated in relative isolation from a complex disease state, using relevant primary cells. Replicate experiments can be performed using primary cells with the same genetic background or cells from different donors¹². Detailed functional and biochemical analyses may then be applied to define potential therapeutic targets^{12,13}.

Filaggrin is a marker of keratinocyte differentiation; it is expressed in the granular layer of the upper epidermis as a polymer – profilaggrin – which undergoes post-translational modification and stepwise proteolysis to release monomeric filaggrin¹⁴. Profilaggrin and filaggrin have multiple functions, each of which have been described as contributing to the mechanical, biochemical, immunological and microbiological aspects of skin barrier function¹⁵. Clinical studies have shown that filaggrin haploinsufficiency is associated with xerosis and ichthyosis (dry and scaly skin), keratosis pilaris (increased keratin within skin follicles), palmar hyperlinearity¹⁶ and increased transepidermal water loss¹⁷. Transcriptomic analysis of full thickness skin biopsies have shown evidence of an abnormal defence response in *FLG* haploinsufficient atopic skin¹⁸; proteomic analysis to assess the effect of *FLG* knockdown in an epidermal organoid has also shown features of inflammation and stress protease activity¹⁹. However, *in vitro* studies have not shown consistent histological or functional effects of filaggrin deficiency in the various different skin organoid models published to date²⁰ and the multiple mechanisms by which filaggrin deficiency contributes to atopic disease remain incompletely understood⁵.

We have optimised a skin organoid model, with dermal and epidermal compartments cultured using donor-matched primary cells, for functional assessments and global mass spectrometry proteomic analysis. The work aims to investigate in more detail the effect of *FLG* siRNA-mediated knockdown on one cell type – the keratinocyte – to increase understanding of the filaggrin-deficient phenotype and to further define molecular mechanisms predisposing to atopic skin inflammation.

Methods

Source of primary human cells

Primary keratinocytes and primary fibroblasts were isolated from human skin tissue samples obtained, with written informed consent and Ethical Committee approval (East of Scotland Research Ethics Service reference 17/ES/0130 renewal 12/ES/0083) under governance of the Tayside Biorepository. Surgical surplus samples of clinically normal skin from four adult donors (all females aged 29–65 years; one abdominal and three breast skin reductions) were used for the organoid cultures. Similar samples (n=5) were used for independent biological replicates to test for reproducibility of the functional effects of *FLG* knockdown.

Organoid culture methods

Primary keratinocytes and dermal fibroblasts were isolated from human skin by sequential trypsin EDTA and collagenase D digestion²¹. Using our previously reported methods¹², the keratinocytes were co-cultured with mitomycin C inactivated 3T3 feeder cells in RM media (3:1 DMEM : Hams F12, 10% FCS, 0.4µg/ml hydrocortisone, 5µg/ml insulin, 10ng/ml EGF, 5µg/ml transferrin, 8.4ng/ml cholera toxin and 13ng/ml liothyronine) (Sigma Aldrich, Gillingham, Dorset, UK)²². Epidermal growth factor (EGF) was omitted for the first day of culture. The fibroblasts were cultured in DMEM supplemented with 10% FCS under standard conditions.

Fibrin gel dermal equivalents¹² were prepared using 0.5ml fibrinogen (35 mg/ml in NaCl) (Sigma Aldrich, Gillingham,

Dorset, UK) and 0.5ml thrombin (3U/ml in 2 mM CaCl_2 / 1.1% NaCl) (Sigma Aldrich, Gillingham, Dorset, UK) combined on ice, with 200,000 fibroblasts and aprotinin (0.1 U/ml) (Sigma Aldrich, Gillingham, Dorset, UK) then transferred to a 12-well plate. After 30 minutes incubation at 37°C, the gels were covered in medium (DMEM, 10% FCS, 0.1 U/ml Aprotinin) and cultured overnight (day 1). On day 2, the medium was replaced with RM excluding EGF, 0.1U/ml aprotinin and 2×10^6 suspended keratinocytes. Culture medium was refreshed daily on days 3 and 4 using RM containing 0.1ng/ml EGF and 0.1U/ml aprotinin. On day 5 the gels were carefully removed from wells and lifted onto custom-made steel grids lined with nylon gauze (Millipore, Livingston, Scotland, UK). RM medium supplemented with 0.1ng/ml EGF and 0.1U/ml aprotinin was added up to the base of the dermal equivalent so that the epidermis

remained at the air-liquid interface. Medium was refreshed on alternate days and the cultures were used for analysis up to day 12 (representing three, five and seven days after lifting to the air-liquid interface).

A diagrammatic summary of the process of skin organoid culture is shown in [Figure 1](#).

Epidermis was separated from the fibrin gel using hypertonic saline-induced split (4 hours, 1M NaCl, 4°C) to obtain a keratinocyte-only tissue sample for biochemical analyses.

FLG genotyping

DNA was extracted from donor skin samples using standard techniques and genotyping was performed for the four

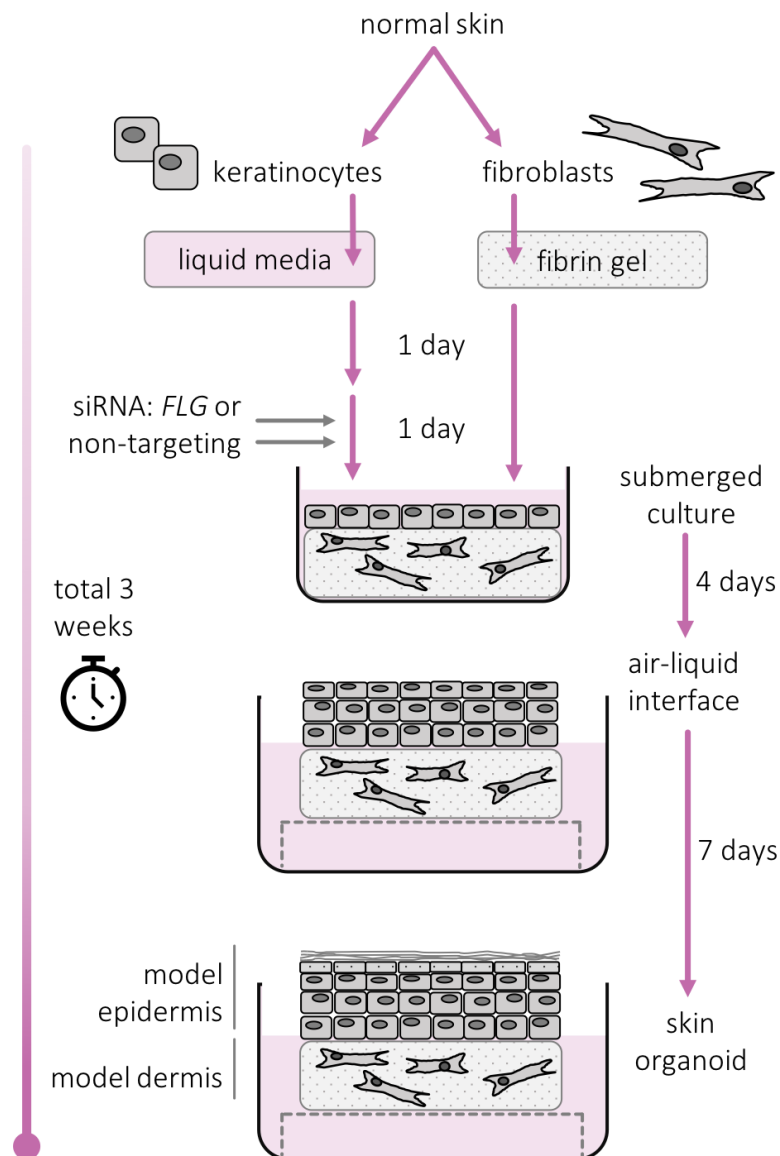


Figure 1. Diagrammatic summary of skin organoid culture. Dermal and epidermal equivalents produced using our published methodology optimised for skin barrier assessment¹².

most prevalent loss-of-function mutations in *FLG* in this white European population (R501X, 2282del4, R2447X and S3247X)²³ using validated KASP™ technology (LGC Genomics, Hoddesdon, Hertfordshire, UK), as previously reported^{4,24}. Briefly, KASP™ SNP genotyping consists of two competitive, allele-specific forward primers and one common reverse primer. Each forward primer incorporates an additional tail sequence that corresponds with one of two universal FRET (fluorescent resonance energy transfer) cassettes (FAM and HEX). ROX™ passive reference dye, Taq polymerase, free nucleotides and MgCl₂ in an optimised buffer solution make up the reaction master mix.

siRNA mediated knockdown

Keratinocytes were reverse transfected immediately prior to inclusion in the organoid cultures using RNAiMax transfection reagent (Life Technologies, Carlsbad, California, USA) according to manufacturer's instructions. Briefly, siRNA complexes were formed in OPTIMEM medium (20μM siRNA, 5μl RNAiMAX) and following 20 minutes incubation, combined with 2×10^6 suspended normal human keratinocytes and transferred to the pre-prepared dermal substrate. A pool of four siRNA duplexes was used [FLG: LQ-021718-00-0002, Control: ON-TARGETplus non-targeting siRNA #4 D.001810-04-20] (Dharmacon, Lafayette, Colorado, USA).

Quantitative PCR

RNA was extracted from organoid epidermis using the Direct-zol RNA kit (R2071, Zymo Research, Irvine, California, USA) following homogenisation in RNA-Bee (CS-104B, Amsbio, Abingdon, Oxfordshire, UK) using the TissueLyser LT (Qiagen, Manchester, UK) 5 mins at 50 Hz. cDNA was prepared from 1μg total RNA using random priming (2.5μM final) (Integrated DNA Technologies, Coralville, Iowa, USA) in combination with Moloney murine leukemia virus (MMLV) enzyme (100 units) and buffer systems (28025013, Life Technologies, Carlsbad, California, USA). qPCR reactions were performed using exon spanning probe-based assays (*FLG*: HS.PT.58.24292320FAM, *EF1A*: HS.PT.58.24345862FAM) [Integrated DNA Technologies, California, USA] with TaqMan gene expression Mastermix (4369016, Life Technologies, Carlsbad, California, USA). Reactions were prepared and run using the Qiagility robot in combination with the Rotor-Gene Q (Qiagen, Manchester, UK). *EF1A* was used as a reference gene. PCR cycling conditions were: 95°C for 10 mins, [95°C for 15 seconds, 60°C for 60 seconds] × 40 cycles. Fold changes were derived via the 2^(-Delta Delta C[T]) method.

Western blotting

Organoid epidermis was homogenized in RIPA buffer (Cell Signalling Technologies, London, UK) using the TissueLyser LT (Qiagen, Manchester, UK) 5 mins at 50 Hz. Protein lysates were normalized by Pierce BCA assay (23225, Thermo Fisher, Waltham, Massachusetts, USA) and resolved under reducing conditions using the NuPage® gel electrophoresis system (Life Technologies, Carlsbad, California, USA). Primary antibodies (monoclonal mouse filaggrin [RRID: AB_1122916, catalogue # sc66192, Santa Cruz Biotechnology, Texas, USA] and monoclonal mouse GAPDH [RRID: AB_2756824, catalogue # 97166,

Cell Signalling Technologies, Boston, USA]) were prepared in 5% bovine serum albumin (BSA) and incubated overnight at 4°C with agitation. Immunoblots were developed using either goat anti rabbit (P0448, RRID: AB_2617138) or goat anti mouse (P0447, RRID: AB_2617137) peroxidase conjugated secondary antibodies (DAKO, Glostrup, Denmark, 1:5000) in combination with chemiluminescent HRP substrate (Immobilon, Millipore, Billerica, Massachusetts, USA) onto photographic film. Densitometry was performed using Fuji ImageJ (May 2017).

Structural assessments

(i) Histology

Formalin-fixed, paraffin embedded full thickness organoids were sectioned and stained using haematoxylin and eosin and imaged using Visiscope LT384P (VWR International, Lutterworth, UK).

(ii) Transmission electron microscopy (TEM)

Organoid skin samples were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) for one hour then cut into small pieces, washed in buffer and post-fixed in 1% osmium tetroxide in cacodylate buffer for one hour or 1% ruthenium tetroxide in cacodylate buffer for one hour. The pieces were dehydrated through alcohol series, into propylene oxide and embedded in Durcupan resin. Ultrathin sections were stained with 3% aqueous uranyl acetate and Reynold's lead citrate and examined on a JEOL 1200EX electron microscope. TEM images were collected on a SIS Megaview III camera.

Functional assessments

(i) Trans-epidermal water loss (TEWL)

Organoid cultures were equilibrated at room temperature and atmospheric conditions for 30 minutes before TEWL was measured at two locations on the epidermal surface using an Aquaflux AF200 instrument (Biox Systems Ltd, London, UK) with a closed chamber and a custom (5mm diameter) probe head. TEWL measurements were taken every second for a minimum of 60 seconds until a stable reading, as determined by the software, was obtained. TEWL directly measures water evaporation from the skin surface but it may be considered as a measurement of 'inside-outside' barrier function²⁵.

(ii) Capacitance

To inform the interpretation of TEWL, we measured electrical capacitance; this is directly proportional to water content of the uppermost 10–20μm of tissue. Organoid cultures were equilibrated at room temperature and atmospheric conditions for 30 minutes prior to measurement of epidermal surface capacitance, using a Corneometer™ and multiprobe adapter (CM825 and MPA2, Courage and Khazaka, Cologne, Germany). Three measurements were recorded from each organoid and the mean was calculated.

(iii) Fluorescent dye penetration

50μl of 1mM lucifer yellow dye (Sigma Aldrich) was added to the epidermal surface of the organoid and incubated at

37°C for 4 hours. Metal cloning rings were used to control uniform dosing on the epidermal surface. The lucifer yellow was removed and the organoids washed in PBS before formalin fix-paraffin embedding under standard conditions. 4µm sections were deparaffinized, counterstained with DAPI (1µg/ml for 10 mins) (Life Technologies, Carlsbad, California, USA) and imaged by confocal Zeiss LSM710 microscope. Quantification of dye penetration in the upper dermis (average intensity in upper 40µm) was performed using Zeiss Zen Blue lite version 2.3 software and compared using paired t-tests. Dye penetration represents a measurement of the 'outside-inside' barrier function.

Mass spectrometry proteomic analysis

Frozen organoid epidermal samples were ground on dry ice, solubilized in 200µl of Cellular and Organelle Membrane Solubilizing Reagent (C0356-4BTL, also called Protein Extraction Reagent Type 4, Sigma-Aldrich, Gillingham, Dorset, UK), 7.0 M urea, 2.0 M thiourea, 40 mM Trizma® base (Sigma Aldrich, Gillingham, Dorset, UK) and 1.0% C7BzO, pH 10.4 buffer with protease inhibitors. The lysate was acetone precipitated for 1 hour at -20°C and spun for 20 mins at 2°C at 15,000 rpm. The pellet was re-suspended in 200µl of 50mM ammonium bicarbonate. 100µl was taken for in-solution digest. 22µl of dithiothreitol was added and samples heated to 50°C for 15mins. 24µl of iodoacetamide was added and the samples incubated at 22°C for 30 mins. 1µl of RapiGest™ (186001860, Waters, Herts, UK) was added, followed by 2.5µl of trypsin, for a 12-hour digest. A further 1µl trypsin was added for an additional 4-hour digest. The peptide samples were fractionated into 24 fractions with high pH Reversed Phase C18 chromatography, run on a Q Exactive Classic or Plus for 160 mins and the top 15 ions selected for sequencing. Mass spectrometry resolution was 70,000 and tandem mass spectrometry (MS/MS) resolution 17,500. Data were processed using MaxQuant (v1.6.0.13) and Human UniProt Database (Dec 2017) with a protein and peptide false discovery rate of 0.01.

Proteins identified as contaminants (trypsin and other lab originating proteins) and contained within the [MaxQuant database](#) were removed. To account for the possibility of keratin as a contaminant, we analysed the blank runs for keratin peptide intensity and subtracted this from the measured keratin intensities. A reversed database was used to identify false positives. The peptide and protein false discovery rate was set at 1%, and these were removed from further analysis.

Total intensities of the samples were normalized to total protein abundance, with adjustment to the lowest total protein yield. All samples were run in quadruplicate and reproducibility was assessed by Pearson correlation.

Mass spectrometry lipidomic analysis

Organoid samples were sonicated in phosphate buffered saline (pH7.4) and then extracted according to the method of Folch *et al.*²⁶. The samples were analysed by liquid chromatography-mass spectrometry (LC-MS) on a Thermo Exactive Orbitrap mass spectrometer (Thermo Scientific, Hemel Hempstead, UK)

coupled to a Thermo Accela 1250 ultra high pressure liquid chromatography (UHPLC) system. Samples were injected on to C18 column (Thermo Hypersil Gold, 2.1 mm x 100 mm, 1.9 µm) and separated using a water/acetonitrile/isopropanol gradient²⁷. Ceramide 17:0 (Avanti Polar Lipids, Alabaster, AL, USA) was included as an internal standard. Ion signals corresponding to individual ceramide molecular species were extracted from raw LC-MS data sets. Concentration of ceramide was expressed as pmol/mg after normalisation to mg of wet weight tissue.

Data analysis of qPCR, immunoblotting and functional assessments

Measurements were compared between non-targeting control-treated organoid models and *FLG*-siRNA-treated models, using paired t-test.

Histological sections of 10 replicate skin organoid experiments were measured using a standardized technique to minimize bias, as follows: three points were measured on each slide, at the right side, middle and left of each sample, across viable cell layers and stratum corneum; measurements were generated using Fuji ImageJ (May 2017) and the mean of the three measurements calculated.

Proteomic data analysis

The total intensities of the samples were normalised to total protein abundance, with adjustment to the lowest total protein yield. Log2 ratio of protein expression (log2 *FLG* knockdown – log2 NT control) was visualised as a volcano plot, using GraphPad Prism (version 5) for human proteins detected in (three or all four) of the replicate experiments. P values were calculated by paired t-test applied to log 10 transformed data. Because of the burden of multiple testing in this large dataset, rather than defining arbitrary thresholds for 'statistical significance', our analyses focussed on changes that showed consistency across the biological replicate experiments. Approximately 1000 of the >8000 proteins were selected as showing consistently increased or decreased abundance, defined as follows:

Proteins were considered to be reduced in abundance if they showed a reduction in all four biological replicate experiments comparing *FLG*-knockdown organoid samples to matched non-targeting controls and ratio ≤0.5 in three or four out of four of the replicates and reduced to undetectable levels of protein in a maximum of two replicates. Proteins were considered to be increased in abundance if they showed an increase in all four biological replicate experiments comparing *FLG*-knockdown organoid samples to matched controls treated with non-targeting controls and ratio ≥1.2 in three or four out of four of the replicates. These magnitudes of change were used to define roughly equal proportions of up- and down-regulated proteins for pathway analysis. The groups were then assessed using gene ontology and network analysis in [STRING](#), version 11²⁸ and pathway prediction using the [Reactome Knowledge-base](#), version 68²⁹. This analysis, based on consistency of findings across multiple biological replicate experiments was used in preference to a defined threshold of statistical significance,

with the aim to identify proteins and pathways of importance in the context of a common complex but heterogeneous trait.

Lipidomic data analysis

The ratio of ceramide and omega-hydroxy ceramide species were compared between the *FLG* knockdown organoids and matched control samples in five biological replicate experiments.

Results

All samples are wild-type for the prevalent *FLG* null mutations and filaggrin shows knockdown at mRNA and protein level

Genotyping of DNA from tissue samples from the four skin donors did not detect any of the prevalent *FLG* null mutations³⁰; this does not, however, exclude the possibility of rare (<0.01 allele frequency) null mutations.

FLG knockdown in the skin organoids persisted to day 10 after siRNA transfection, as demonstrated by qPCR (Figure 2) and western blotting (Figure 3). We note the greater magnitude of knockdown quantified at mRNA than protein level in the organoid epidermis 10-days after transfection. This is likely to reflect the inherent differences in mRNA and protein stabilities: *FLG* mRNA has a half-life of ~2.2 hours³¹ whereas the half-lives of profilaggrin and filaggrin are approximately 6 and 24 hours respectively³².

FLG knockdown results in morphological changes in the epidermis

Histological examination confirmed a reduction of the granular layer, in keeping with a reduction in profilaggrin (Figure 4A). There was also a slight reduction in thickness

of the stratum corneum (ratio 0.83 ± 0.05 (mean ± SEM), but no difference in thickness of the keratinocyte cell layers (Figure 4B).

Transmission electron microscopy (TEM) of the skin organoid model shows an effective recapitulation of the physiological layers of keratinocyte differentiation within skin, including a mature, multi-layered stratum corneum with corneodesmosomes and a granular layer with keratohyalin granules and desmosomes (Figure 5A). TEM of the *FLG*-knockdown organoid shows a similarly mature epidermis, but the stratum corneum has fewer layers and the granular layer contains keratohyalin

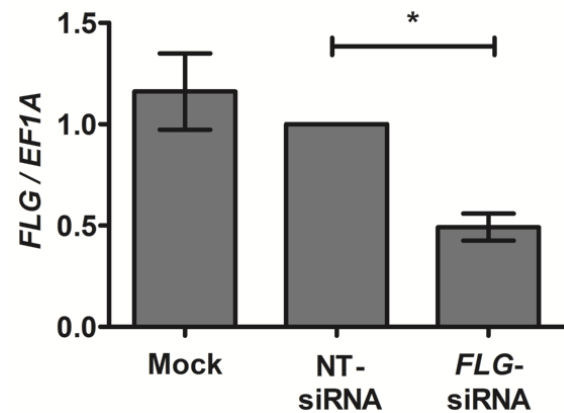


Figure 2. qPCR analysis of epidermis from skin organoids with and without *FLG* siRNA-mediated knockdown. Probes and primers are listed in Methods; n=8 biological replicates; error bars show SEM; *paired t-test p<0.05.

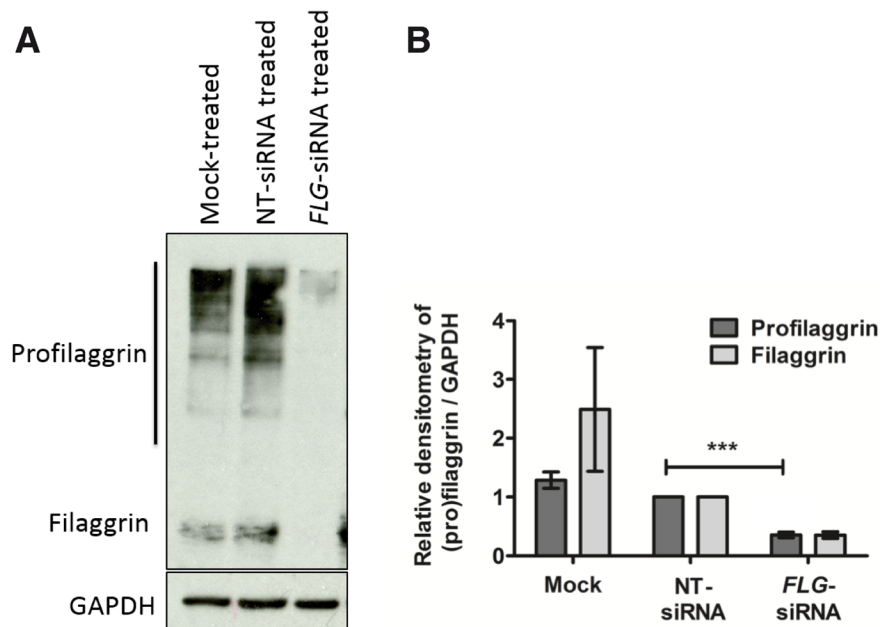


Figure 3. Quantification of filaggrin in organoid epidermis. (A) Representative western blot. (B) Approximated quantification by densitometry. NT, non-targeting; n=7; ***p<0.0005 compared to non-targeting control.

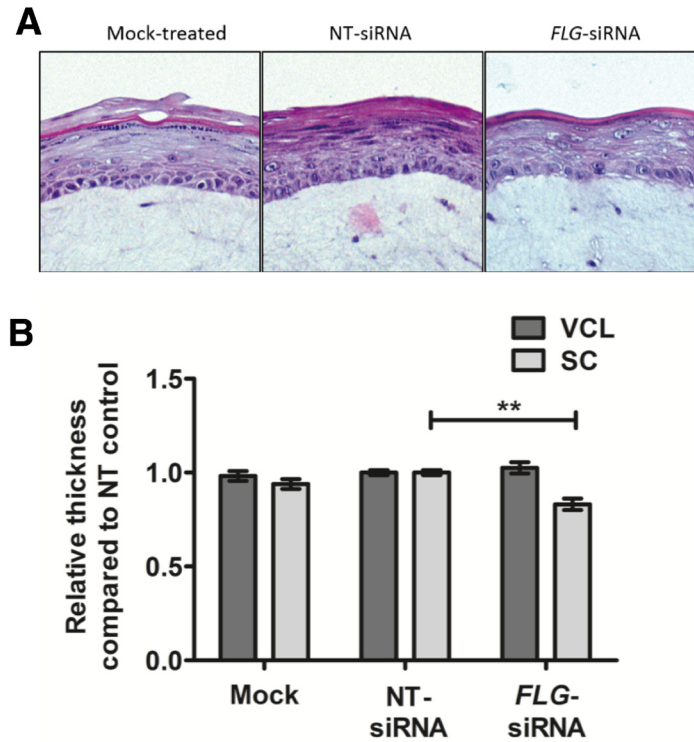


Figure 4. Structural changes in organoid models. (A) Histological examination (representative images from 8 biological replicates). NT, non-targeting. (B) Measurement of relative thickness of epidermal layers: VCL, viable cell layer and SC, stratum corneum; n=8; compared using paired t-test; **p<0.005.

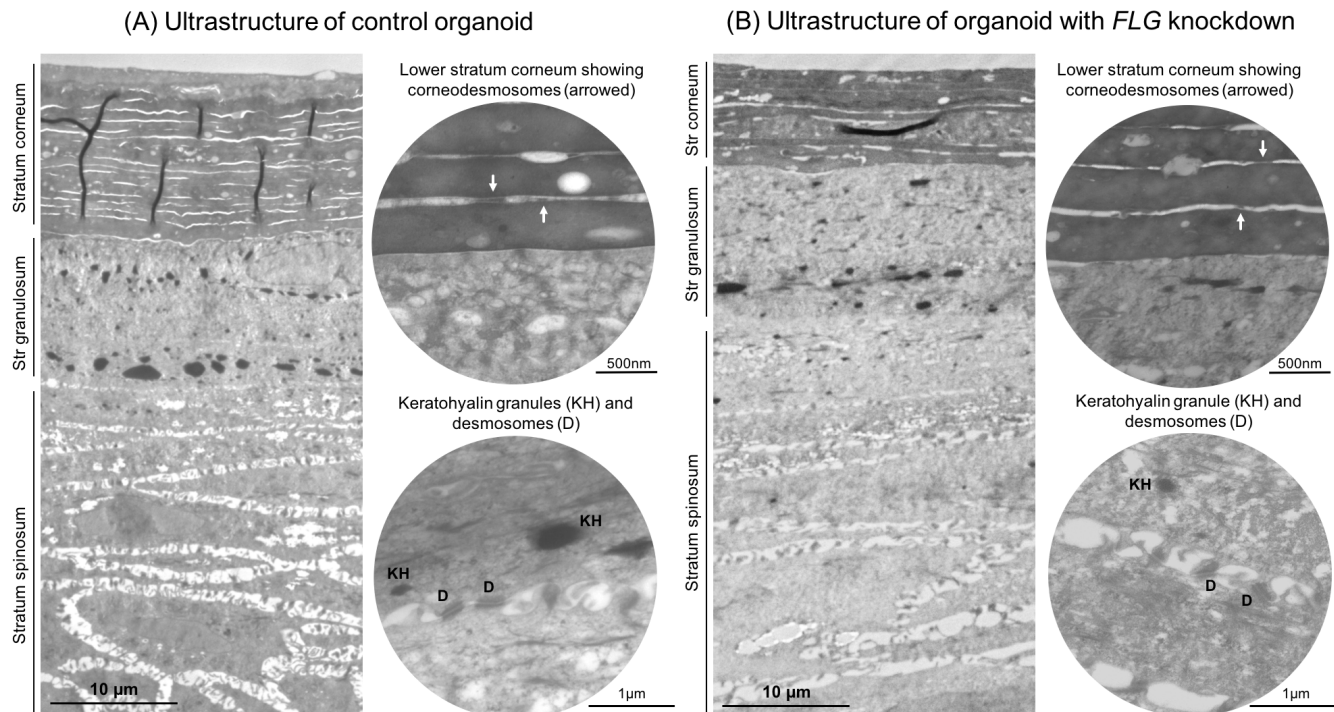


Figure 5. Ultrastructural features of control and FLG knockdown organoids. (A) Control organoid sample, showing elements of normal human skin structure including stratified differentiation. (B) Organoid with FLG siRNA-mediated knockdown, showing thinner stratum corneum, reduced prominence of the corneodesmosomes and desmosomes, and smaller keratohyalin granules (organelles containing profilaggrin).

granules that are generally smaller and less numerous than in the control organoid (Figure 5B).

FLG knockdown shows some evidence of a barrier defect in the immature organoids but no functional effect on skin barrier in the mature organoid model

The organoid model described here shows progressive development of skin barrier function after lifting to the air-liquid interface: there is a progressive reduction in TEWL (Figure 6), progressive reduction in capacitance (Figure 7) and the hydrophilic dye, lucifer yellow, is excluded from the epidermis by day 7 in both the control and *FLG*-knockdown organoids (Figure 8).

Results from five biological replicate experiments showed there was no difference in the mean TEWL (Figure 6), or mean capacitance measurements (Figure 7) between the mature organoid models with *FLG* knockdown and donor-matched non-targeting siRNA-treated controls. Capacitance was increased in the immature

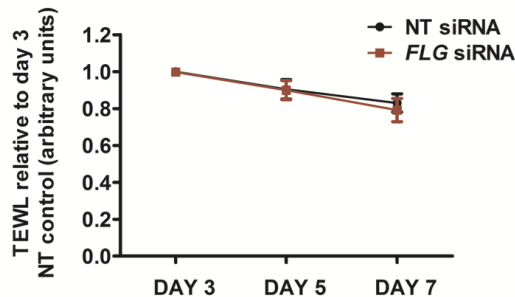


Figure 6. Transepidermal water loss (TEWL) measurements. TEWL measured under standard conditions at days 3, 5 and 7 after the organoid is lifted to the air-liquid interface, using an Aquaflux AF200 instrument (Biox Systems Ltd, London, UK) with a 5mm diameter probe head. NT, non-targeting; N=5; error bars show SEM.

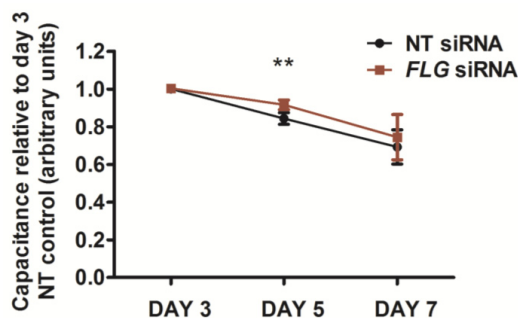


Figure 7. Epidermal capacitance measurements. Electrical capacitance, directly proportional to water content of the uppermost 10–20µm of tissue, was measured under standard conditions at days 3, 5 and 7 after the organoid is lifted to the air-liquid interface, using a Corneometer™ (Courage and Khazaka, Cologne, Germany); the mean of three measurements is recorded. NT, non-targeting; N=5; error bars show SEM; paired t test **p<0.005.

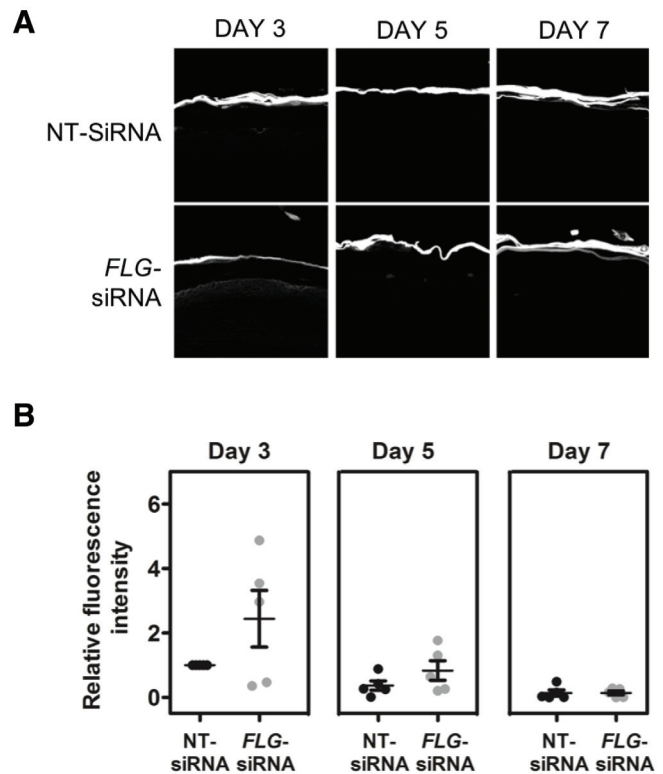


Figure 8. Lucifer yellow dye penetration of organoid samples. The hydrophilic lucifer yellow dye (Sigma Aldrich) was added to the epidermal surface of the organoid and incubated at 37°C for 4 hours before sectioning for microscopic examination. (A) Representative image from three of the five biological replicate experiments showing delayed maturation of barrier function. (B) Dot-plot of densitometry data from all five biological replicates. NT, non-targeting.

FLG-knockdown model (day 5 after air-exposure, Figure 7) and lucifer yellow dye was not fully excluded by the stratum corneum in some of the the *FLG*-knockdown replicate experiments until day 7 (Figure 8). However, this delay in maturation of the ‘outside-to-inside’ barrier function was not consistently demonstrated in all biological replicates.

Mass spectrometry proteomic analysis identifies over 8000 protein species per sample

After removal of contaminants and false positive results, >8000 proteins were identified in each epidermal sample. The proteomic data have been deposited to the ProteomeXchange Consortium via the PRIDE³³ partner repository with dataset identifier PXD014875. Quality control analysis confirmed that similar distributions of proteins were identified in each experiment (Figure 9A); protein extracts were analysed in quadruplicate to test for technical reproducibility and the Pearson correlations were 0.869–0.938 (Figure 9B). The protein expression changes were visualised using a volcano plot (Figure 10).

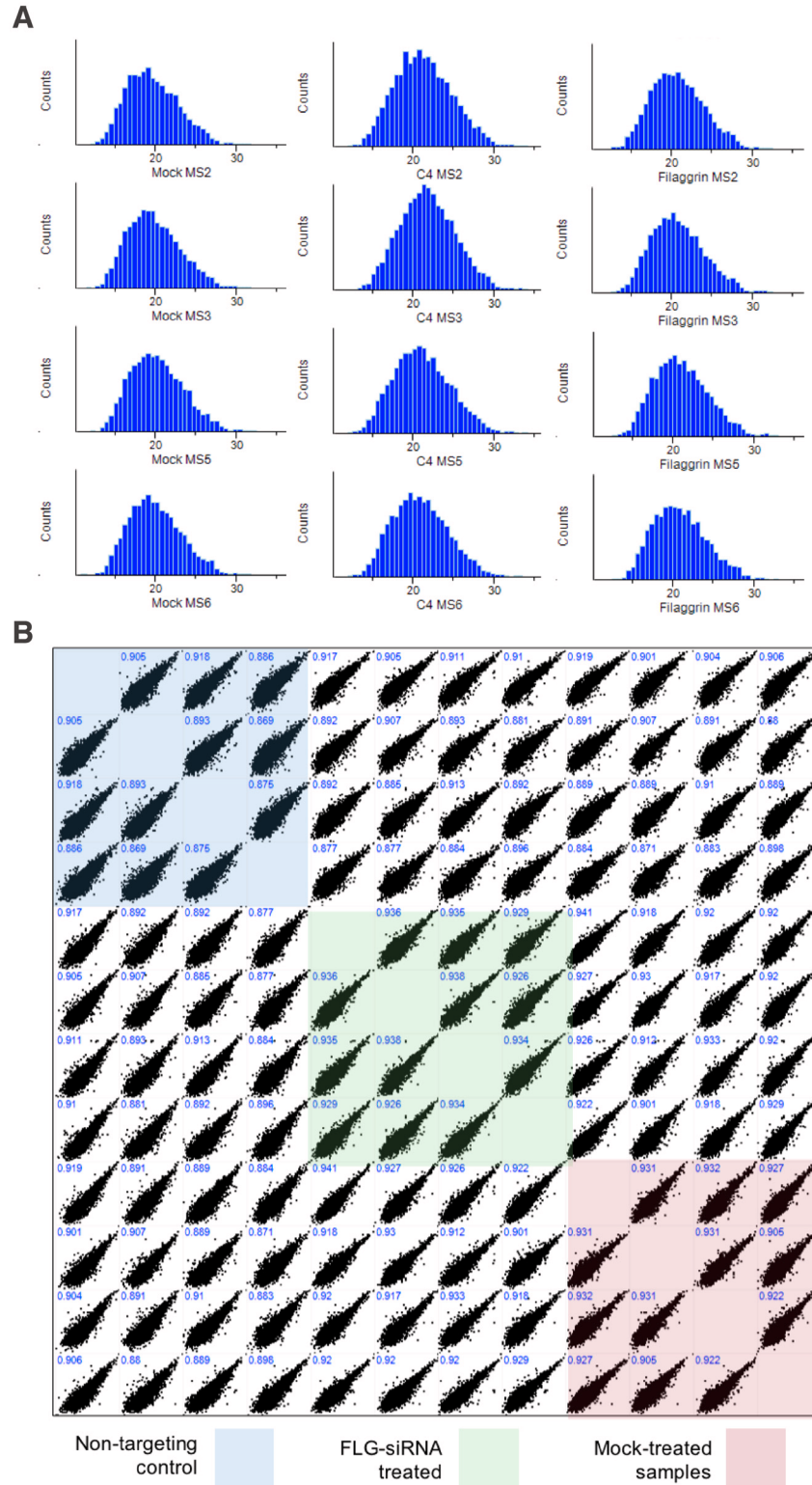


Figure 9. Mass spectrometry proteomic analysis quality control assessments. (A) Histograms showing the distribution of proteins quantified in each experiment. Bins on the x-axis contain the log₂ intensity-based absolute quantification (iBAQ, sum of intensities of tryptic peptides) for each protein divided by number of theoretically observable peptides; normalised counts of the proteins in each bin are shown on the y-axis; MS2, MS3, MS5 and MS6 are biological replicate experiments from different human donors; mock, untreated control organotypic; C4, non-targeting control treated organotypic; Filaggrin, FLG si-RNA treated organotypic. **(B)** Correlation of protein abundance from replicate analyses. x and y axes show iBAQ intensities of samples mapped against replicate analysis; biological replicates are highly correlated (Pearson correlation coefficient ≥ 0.86).

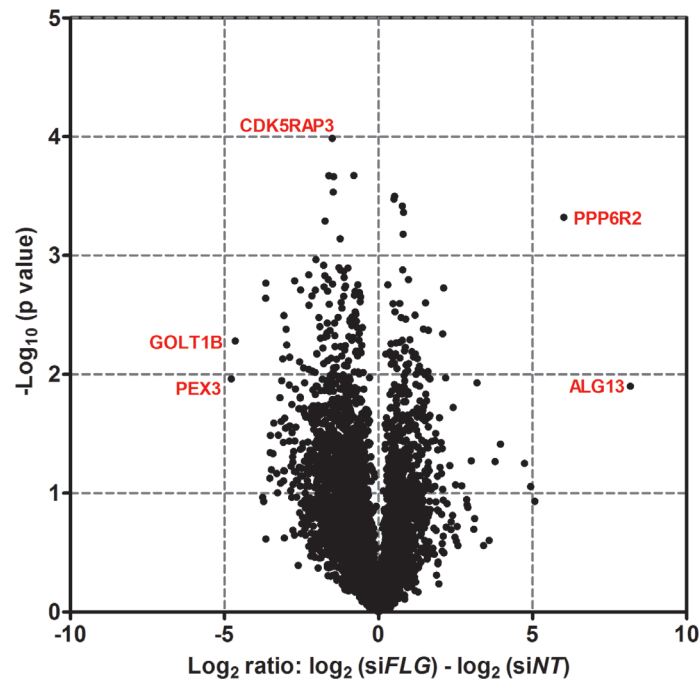


Figure 10. Volcano plot to visualise changes in protein expression between control and FLG siRNA-treated organoids. Adjusted protein intensity data were filtered to include only human proteins detected in three or four replicate experiments. The log₂ ratio (log₂ FLG knockdown – log₂ NT control) for each donor was calculated and averaged; p-values from paired t-test were derived from log₁₀ transformed data and are unadjusted. Selected data-points with more extreme values are labelled using the abbreviated protein name. NT, non-targeting.

648 proteins show a consistent reduction in expression in skin organoids with FLG knockdown

648 human proteins showed a reduction in expression in all four of the replicates and ratio ≤ 0.5 in three out of four replicates. This list is available as *Underlying data*, uploaded to Figshare³⁰.

645 of the proteins are identified in STRING and this group showed significant enrichment for interactions ($p < 1.0 \times 10^{-16}$) computed by combining the probabilities from the different evidence channels and corrected for the probability of randomly observing an interaction²¹. Gene ontology (GO) analysis in STRING showed evidence for functional enrichment of GO-terms including RNA processing (false discovery rate (FDR) 4.40×10^{-11}), catalytic activity (FDR 8.59×10^{-8}) and intracellular component (FDR 3.88×10^{-26}). A list of the 10 most significant GO analysis results in each of the GO domains is shown in Table 1 (see *Underlying data*)³⁴.

Pathway analysis of down-regulated proteins identifies themes including RNA metabolism, adaptive immunity and axon guidance

Reactome detected 39 pathways in this set of proteins (Table 2, *Underlying data*)³⁵. Descriptions include: metabolism of RNA (including 67 out of the 652 genes in this pathway, FDR 3.36×10^{-12}); class I MHC mediated antigen processing and presentation (30/365 genes in pathway, FDR 0.0031); and axon guidance (34/541 genes, FDR 0.0207).

Down-regulated proteins indicate molecular mechanisms of relevance to atopic eczema

Manual searching of the list of down-regulated proteins identified several of specific interest based on *a priori* knowledge of eczema pathomechanisms. These highlight several putative therapeutic targets: the aryl hydrocarbon receptor plays a role in signalling pathways for skin barrier repair²² and has been proposed as a therapeutic target for the treatment of skin inflammation³⁶; caspase 14 plays a role in filaggrin processing³⁷ and is required for epidermal protection against water loss and UVB-induced damage³⁸; dermatokine is a soluble regulator of keratinocyte differentiation³⁹; sequence similarity to a murine protein (UniProtKB:Q8R1R3) indicates that StAR-related lipid transfer protein 7 may play a role in protecting mucosal tissues from exaggerated allergic responses; a reduction in the cytokine TGF-beta 1 may predispose to atopic eczema⁴⁰; and loss-of-function mutations in *CYP4F22* cause lamellar ichthyosis⁴¹, suggesting that a reduction in CYP4F22 protein expression may contribute to the ichthyotic phenotype observed in ichthyosis vulgaris and atopic skin.

376 proteins show a consistent increase in expression in organoids with FLG knockdown

376 human proteins showed an increase in expression in all four of the replicates and ratio ≥ 1.2 in three or four out of the four replicates. This list is available as *Underlying data*, uploaded to Figshare³⁰.

374 proteins are identified in STRING and the group showed significant enrichment for interactions ($p < 1.0 \times 10^{-16}$). GO analysis revealed enrichment of terms including translation (FDR 4.12×10^{-19}), RNA binding (FDR 9.77×10^{-12}) and cytoplasmic part (FDR 9.64×10^{-36}). A list of the 10 most significant GO analysis results in each of the GO domains is shown in Table 3 (see *Underlying data*)⁴².

Pathway analysis of up-regulated proteins identifies themes including translation, innate and adaptive immunity and axon guidance

Reactome detected 113 pathways in this set of proteins with FDR $p < 0.05$ (Table 4, *Underlying data*)⁴³. Descriptions include: Translation (including 44 out of the 288 genes in this pathway, FDR 1.17×10^{-21}); innate immune system (47/1012 genes in pathway, FDR 9.90×10^{-7}); cytokine signalling in immune system (32/654 genes in pathway, FDR 5.16×10^{-5}); and axon guidance (40/541, FDR 4.69×10^{-11}).

Up-regulated proteins indicate molecular mechanisms of relevance to atopic eczema

Manual searching of the list of up-regulated proteins identified several of relevance to atopic eczema, including: STAT1, an intracellular signalling molecule that shows increased expression in atopic eczema, and as part of the JAK-STAT pathway is targeted by several traditional herbal remedies⁴⁴ as well as novel small molecule therapies⁴⁵; carbonic anhydrase 2, which is increased in skin in a variety of forms of eczema⁴⁶; and FK binding protein, which binds to tacrolimus, an established topical treatment for atopic eczema.

The type I keratins 10, 16 and 17 each show increased expression in the *FLG*-siRNA-treated organoid models with mean ratio 1.7, 1.3 and 1.3, respectively. Keratin 10 is an intermediate filament protein of structural importance in the skin; keratin 16 is an epidermis-specific keratin that plays a role in innate immunity in response to skin barrier breach⁴⁷; keratin 17 is expressed in epidermal appendages, including the hair follicle.

Finally, it is noteworthy that GAPDH, which is often considered to be a stable 'housekeeping' gene, is in the dataset of consistently up-regulated proteins. However, in the context of this work, filaggrin knockdown is clearly apparent at a greater magnitude than the more subtle change in GAPDH (Figure 3).

Epidermal ceramide content shows no significant difference in *FLG*-knockdown organoids compared with controls

Ceramide and omega-hydroxy ceramide species in five biological replicate experiments showed no consistent difference between the *FLG* knockdown organoids and matched control samples (Figure 11).

Discussion

The functional and biochemical analyses of a filaggrin-deficient skin model have provided findings that are consistent with our and others' previous analyses *in vitro*¹⁹ and *in vivo*⁴⁸. We have

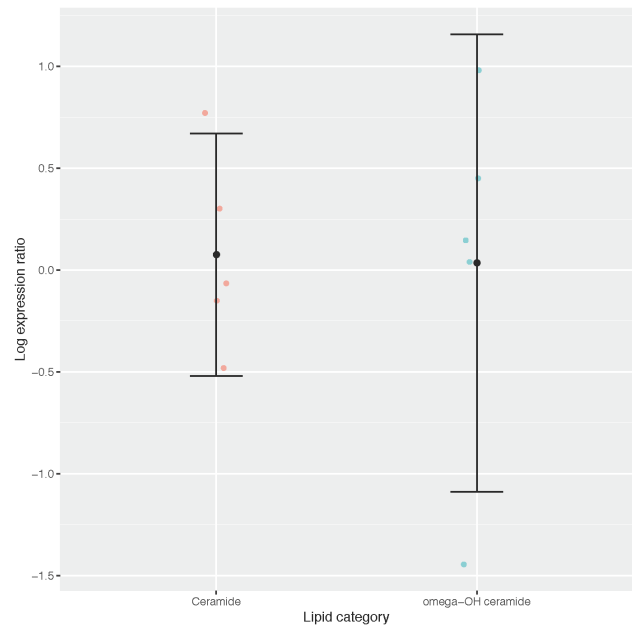


Figure 11. Ratio of ceramides and omega-hydroxy ceramides in organoid epidermis. Dots represent values for five individual experiments; bars show 95% confidence interval.

included both epidermal and dermal compartments in this model (to allow for dermal-epidermal cross-talk^{49,50}) and our more detailed proteomic analysis has revealed additional evidence of molecular mechanisms with relevance to atopic skin. These datasets are shared as a resource for the research community.

Analysis of the immature organoid identified a delay in epidermal maturation following *FLG* knockdown, but the lack of a replicable functional effect in our model, when mature, reflects previous reports in which epidermal equivalents created from *FLG*-null keratinocytes do not show impairment in barrier function²⁰. This is in keeping with the observation that *FLG*-null mutations are not fully penetrant with respect to ichthyosis vulgaris or atopic eczema^{16,51}.

Because of the nature of our data, generated using biological replicate samples, and the focus on modelling a common complex trait with considerable inter-individual variation, we elected to use the criterion of consistency across the biological replicates to define proteins of interest, followed by network and pathway analyses. We recognise that other, more complex data analytical approaches may be applied, and/or more focussed interrogation of specific pathways; we share the proteomics data and welcome such further analysis.

Our analysis has highlighted aspects of keratinocyte-immune signalling. This is an important component of the immunological barrier that may be overlooked in tissue with multiple different cell types. The predicted pathways entitled 'innate immunity' and 'adaptive immunity' included proteins showing

both increased and decreased expression (Table 2 and Table 4, *Underlying data*)^{35,43}. This immune dysregulation is likely to contribute to the predisposition to infection and inflammation observed in filaggrin-deficient skin *in vivo*: filaggrin-deficient patients show increased viral infection of the skin⁵² and increased incidence of irritant^{53,54} and allergic contact dermatitis⁵⁵. The regulation of innate and adaptive immune pathways within keratinocytes (in addition to cells of the haematopoietic lineage) may therefore represent a worthwhile target for future therapeutic interventions.

The lack of effect of *FLG* knockdown on the ceramide content in the model epidermis is also consistent with previously reported findings *in vitro*^{56,57} and *in vivo*⁵⁸. We did not analyse in detail the other epidermal lipids and therefore we cannot comment on whether there were relevant changes. Differences in lamellar body structure⁵⁹ have been reported in *FLG*-mutant skin biopsies but this was not clearly observed in the organoid model. However, ultrastructural examination of the model stratum corneum did reveal abnormalities in the lamellar bilayer structure and less prominent corneodesmosomes (Figure 5), as previously reported *in vivo*⁵⁹. It is also noteworthy that pathway analysis in Reactome identified the terms ‘metabolism of lipids’ and ‘phospholipid metabolism’ from the group of consistently down-regulated proteins (Table 4, *Underlying data*)⁴³.

The most significant themes identified in the groups of proteins showing increased expression upon *FLG* knockdown relate to RNA binding and translation; this is in contrast to RNA metabolism and RNA processing, which are demonstrated in the proteins showing decreased expression. The functional significance of this increase in transcriptional and translational activity cannot be determined from the gene ontology and pathway analysis. We may hypothesise that it reflects the increased ‘stress’ response, as we have previously observed in *FLG* genotype-stratified transcriptome analysis of atopic skin biopsies¹⁸ and/or the activation of innate and adaptive immune mechanisms.

It is tempting to speculate that the increased expression of keratins 10 and 16 may contribute to palmar hyperlinearity and keratin 17 may contribute to keratosis pilaris. These are characteristic features of ichthyosis vulgaris but their pathogenesis has not yet been explained as a direct result of filaggrin haploinsufficiency^{16,60}.

Several of the proteins showing reduced expression relate to aspects of differentiation, including filaggrin itself, and caspase 14 which plays a role in the terminal degradation of filaggrin³⁷. The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor that responds to multiple different environmental and metabolic stimuli to control transcriptional pathways of relevance to atopic eczema, including immunity and differentiation⁶¹. Activation of AhR reduces skin inflammation⁶² and enhances barrier repair⁶³; therefore, a reduction in AhR expression in the *FLG* knockdown organoid would be anticipated to increase the eczema diathesis by opposing these effects. AKT1 activity is known to be reduced in atopic skin, leading

to an alteration in protease expression and reduced filaggrin expression and processing⁶⁴. This mechanism may further exacerbate the filaggrin deficiency in our *FLG* knockdown organoid. Dermokine acts as a soluble regulator of keratinocyte differentiation and mice deficient in the epidermal dermokines (beta and gamma isoforms) show defective cornification⁶⁵. Transforming growth factor (TGF) beta-1 is a multifunctional protein: it regulates the growth and differentiation of multiple cell types, including keratinocytes and it can promote either Th17 or T-regulatory cell lineage differentiation in a concentration-dependent manner; it appears to have an immunosuppressive effect in atopic disease since a genetically-determined lower production of TGF-beta-1 is associated with increased risk of atopic dermatitis⁴⁰.

The Reactome term ‘axon guidance’ was detected in proteins showing increased and decreased expression. It has long been recognised that there is an increase in the density of sensory neurons in eczematous skin⁶⁶ and nerve growth factor expression is upregulated in the keratinocytes of patients with atopic eczema⁶⁷. Therefore, dysregulation of axon guidance may be of relevance to the pruritus that is so characteristic of atopic eczema and contributes substantially to the morbidity of this condition³. Antagonists of transient receptor potential vanilloid subfamily member 1 (TRPV1), expressed on sensory nerves and keratinocytes, are now being investigated for the control of pruritus^{68,69}.

Taken together, our data demonstrate that filaggrin haploinsufficiency leads to abnormalities in both structural and immune features within the keratinocyte compartment of the epidermis. Keratinocytes display immune activity, producing multiple cytokines and chemokines in addition to their roles in innate immunity^{70,71}. Together with the evidence of dysregulation in axon guidance, these findings indicate that patients with filaggrin deficiency – either haploinsufficiency or a reduction in filaggrin expression that occurs secondary to atopic inflammation⁷² – may derive clinical benefit from future therapies targeting keratinocyte-immune crosstalk mechanisms and neurogenic pruritus.

The skin organoid model described here represents a valuable opportunity to study genetic effects on keratinocytes in relative isolation. However, the lack of other cell types (notably T cells, B cells, dermal dendritic cells and innate lymphoid cells), the absence of complex dermal structures (including neurons and blood vessels) and skin appendages (for example hair follicles, sweat and sebaceous glands) is also a limitation in that it precludes assessment of the complex interactions that are likely to occur between different tissue compartments in skin.

In conclusion, we share these experimental results in detailed form, because of the additional insight provided by this *in vitro* analysis of filaggrin deficiency in a skin organoid model; our findings compliment and extend previous work *in vitro* and *in vivo*. Our analyses have re-emphasised the role of keratinocyte-specific mechanisms in contributing to immune and

neurological as well as structural aspects of skin barrier function and offer new insight into molecular mechanisms for the ichthyosis vulgaris phenotype.

Data availability

Underlying data

Mass spectrometry proteomics data deposited in the ProteomeXchange Consortium via the PRIDE partner repository³³, Accession number PXD014875: <https://identifiers.org/ptide.project:PXD014875>

Figshare: Proteomic analysis of *FLG* knockdown in skin organoid. <https://doi.org/10.6084/m9.figshare.c.4606052.v3>³⁰.

This project contains the following underlying data:

- TEM images (zip file containing raw, unedited TEM images in .tif format)
- TEWL and corneometer readings (raw readings in .xlsx format)
- Western blots (zip file containing unedited western blot scans, plus an annotated composite image with labels in .tif format)
- Lucifer yellow dye penetration quantification.xlsx
- Lucifer yellow dye penetration (zip file containing raw, unedited microscopy images for five replicates in .czi format)
- Histology quantification (raw histology quantification data in .xlsx format)
- H&E images (raw, unedited histology images for replicates 1-11 in .bmp and .tif format)
- Filaggrin densitometry (densitometry measurements for seven replicate experiments in .xlsx format)

- FLG qPCR data (raw qPCR data in .xlsx format)
- FLG genotyping results (raw genotyping results in .xlsx format)
- Consistently down-regulated proteins in FLG kd organoids. xlsx
- Consistently up-regulated proteins in FLG kd organoids. xlsx

Figshare: Table 1. Gene Ontology (GO) analysis of proteins showing a consistent reduction in expression with *FLG* knockdown. <https://doi.org/10.6084/m9.figshare.9710585.v1>³⁴.

Figshare: Table 2. Reactome pathway analysis of down-regulated proteins. <https://doi.org/10.6084/m9.figshare.9710666.v1>³⁵.

Figshare: Table 3. Gene Ontology (GO) analysis of proteins showing a consistent increase in expression with *FLG* knockdown. <https://doi.org/10.6084/m9.figshare.9710738.v1>⁴².

Figshare: Table 4. Reactome pathway analysis of up-regulated proteins. <https://doi.org/10.6084/m9.figshare.9710783.v1>⁴³.

Data are available under the terms of the [Creative Commons Attribution 4.0 International license](#) (CC-BY 4.0).

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Reviewer Report 26 November 2019

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Ulrich auf dem Keller

Technical University of Denmark, Kongens Lyngby, Denmark

Sabine Werner

Institute for Molecular Health Sciences, Department of Biology, ETH Zurich (Swiss Federal Institute of Technology Zürich), Zürich, Switzerland

The authors have appropriately responded to the comments of the reviewers and the manuscript is further improved. The purpose of the publication has been further clarified and I understand that the authors prefer not to include validations into this article.

In general, this is a very valuable dataset that will be of interest to researchers in the field of inflammatory skin diseases.

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Skin Biology, Inflammatory Skin Disease, Wound Healing, Tissue Repair

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 09 October 2019

<https://doi.org/10.21956/wellcomeopenres.16840.r36479>

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**Sanja Kezic**

Amsterdam UMC, Coronal Institute of Occupational Health, Amsterdam Public Health research institute, University of Amsterdam, Amsterdam, The Netherlands

Elias *et al.* investigated the effect of filaggrin deficiency in an optimized skin organoid model. Next to the inside/out and outside/in barrier function assessed respectively by TEWL and penetration of a hydrophilic dye, a global mass proteomic analysis has been done. As discussed by authors, the used model has important advantage that the changes related to filaggrin deficiency could be studied in specific cells (keratinocytes) and both epidermal and epidermal compartments were present. On the other side, the model misses other relevant cell types. The study is well designed and presented.

Specific points**1. Skin barrier function assessment**

Although Lucifer yellow is a commonly used model penetrant it has to be realized that owing to its hydrophilicity and a large molar mass (444 Da) it has quite low penetration rate and a long lag time. The incubation time of 4 hours before microscopic examination is likely too short to detect penetration across the SC unless the barrier damage is substantial. In general, it would be good to include in functional studies both, hydrophilic and lipophilic penetrants and measure their penetration at different time points. This might provide more detailed insight into alterations in the structure and composition of the SC and relate them to the structural changes. As noted by the authors, the Flg-knock down “reveal various abnormalities: in the lamellar bilayer structure and less prominent corneodesmosomes” and furthermore down-regulation of proteins involved in metabolism of lipids’ and ‘phospholipid metabolism” and thinner stratum corneum. Though, no changes in either TEWL or dye penetration have been found. This model would be of great value to further study functional consequences of filaggrin deficiency, including previous damage of the skin e.g. by skin irritants.

2. Other points

The authors note that “FLG knockdown in the skin organoids persisted to day 10 after siRNA transfection, as demonstrated by qPCR (Figure 2) and western blotting (Figure 3)”. In Fig. 2 it is obvious that the knock-down at the RNA level was not complete and lower than achieved in similar studies (van Dongelen *et al.*, 2013¹, Wang *et al.*, 2017²). On which day was the mRNA expression measured and what might be reason for quite a low knock-down efficiency?

Ceramide analysis has been carried out in the model epidermis. Performing ceramide analysis in the isolated stratum corneum might have been more relevant.

References

1. van Drongelen V, Alloul-Ramdhani M, Danso MO, Mieremet A, Mulder A, van Smeden J, Bouwstra JA, El Ghalbzouri A: Knock-down of filaggrin does not affect lipid organization and composition in stratum corneum of reconstructed human skin equivalents. *Exp Dermatol.* 2013; **22** (12): 807-12 [PubMed Abstract](#) | [Publisher Full Text](#)
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Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Skin barrier in inflammatory skin diseases, dermatotoxicology.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 19 Nov 2019

Sara J. Brown, Ninewells Hospital, Dundee, UK

Specific points

1. Skin barrier function assessment

We agree that Lucifer yellow has a slow penetration rate hence our long incubation (4 hours, compared to some other publications reporting 1 hour or less). We will, in future work, consider use of a hydrophobic dye as an additional, complimentary assessment - thank you for this useful suggestion.

2. Other points

The mRNA quantification was carried out 10 days after siRNA transfection and we agree it is interesting to note this decay in knockdown at transcriptome level, whilst the protein knockdown persists to a greater degree. This is likely to reflect the different half-lives of *FLG* mRNA (approximately 2.2 hours¹) whereas the half-lives of profilaggrin and filaggrin are approximately 6 and 24 hours respectively.² We have added this additional explanation to the updated manuscript.

Thank you for the knowledgeable advice about ceramide analysis. We agree that the stratum corneum is the most relevant tissue layer in which to perform lipidomic analysis, but an accurate separation of the whole stratum corneum (without upper epidermal cells) proved to be technically

challenging in the organoid model. We therefore chose to analyse full thickness epidermis to ensure consistency between the replicate experiments.

References

1. Nirunsuksiri, W., Zhang, S.H. & Fleckman, P. Reduced stability and bi-allelic, coequal expression of profilaggrin mRNA in keratinocytes cultured from subjects with ichthyosis vulgaris. *J Invest Dermatol* **110**, 854-61 (1998).
2. Markova, N.G. *et al.* Profilaggrin is a major epidermal calcium-binding protein. *Mol Cell Biol* **13**, 613-25 (1993).

Competing Interests: No competing interests were disclosed.

Reviewer Report 01 October 2019

<https://doi.org/10.21956/wellcomeopenres.16840.r36429>

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Neil Rajan 

Institute of Genetic Medicine, Newcastle University, Newcastle upon Tyne, UK

Elias *et al.* present data on a manuscript titled "Proteomic analysis of a filaggrin-deficient skin organoid model shows evidence of increased transcriptional-translational activity, keratinocyte-immune crosstalk and disordered axon guidance".

In this study, primary keratinocytes from 4 female filaggrin wild-type genotyped individuals were subject to siRNA *FLG* kd and a nontargeting siRNA control. These were grown in a skin equivalent model with matched donor fibroblasts, and then keratinocytes alone were harvested after the model had been in at the air liquid interface for 7 days. This was used to carry out proteomic and lipidomic analysis. Additional samples were used for validation work.

The work has been meticulously described in the methods and is an exemplar for human primary keratinocyte research. Filaggrin knockdown was demonstrated at the transcriptomic and protein level, and is evident in the mass spec data across all samples. A phenotype is seen on TEM and is elegantly presented Fig 5. Barrier function as assessed by TEWL and dye penetration is not impaired following *FLG* knockdown at Day 7, which is consistent with prior reports using skin equivalent models.

The key findings are the differentially expressed proteins in this novel model, and unlike prior skin equivalent papers, here dermal fibroblasts are used, allowing for better modelling of human skin where cross talk happens. Notably, lipidomic studies did not show a difference in epidermal ceramides.

Comments:

1. The authors highlight the proteins that have relevance to the known pathogenetic mechanisms of eczema. It would be helpful to emphasise the novel findings detected in this model compared to

the prior proteomic analysis published on filaggrin knockdown keratinocytes (Elias *et al.*, 2017¹), as that model did not include matched donor fibroblasts.

2. Table 2 presents Reactome data and highlights Axon guidance, and it would be helpful to see this in the context of the other pathways described. I would favour this being included in the PDF, rather than Fig 9, which may be moved to supplementary data.
3. The volcano plot (Fig 10) is of interest, but more data could be presented here, for example, the 7 points which are log2 fold change >+5 could be identified to make this more informative for the reader. I am uncertain if the colour scheme with p value cut offs is helpful; the authors carefully justify using selected fold change thresholds in the methods section over arbitrary thresholds that are p-value driven and this depiction is at odds with this.
4. Could the title be improved to reflect the more complete nature of this skin equivalent model that incorporates fibroblasts? Also, how were the three elements "increased transcriptional-translational activity, keratinocyte-immune crosstalk and disordered axon guidance" selected or prioritised from the list of Reactome findings?

There is a minor typographical error on page 3. "Medium was refreshed on alternate day and the cultures were used for analysis up to day 12 (representing three, five and seven days after lifting to the air-liquid interface)".

References

1. Elias MS, Long HA, Newman CF, Wilson PA, West A, McGill PJ, Wu KC, Donaldson MJ, Reynolds NJ: Proteomic analysis of filaggrin deficiency identifies molecular signatures characteristic of atopic eczema. *J Allergy Clin Immunol.* 2017; **140** (5): 1299-1309 [PubMed Abstract](#) | [Publisher Full Text](#)

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Dermatology, Genetics, Primary cell culture models.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 19 Nov 2019

Sara J. Brown, Ninewells Hospital, Dundee, UK

Comments:

The authors highlight the proteins that have relevance to the known pathogenetic mechanisms of eczema. It would be helpful to emphasise the novel findings detected in this model compared to the prior proteomic analysis published on filaggrin knockdown keratinocytes (Elias et al., 20171), as that model did not include matched donor fibroblasts.

Thank you for this insightful comment. The current work differs in several ways from the previous work (performed by the same first author in different research institutions):

- (i) The current work used FLG wild-type primary keratinocytes isolated from female breast skin and co-cultured with 3T3 feeder cells in the presence of serum, whereas the previous publication used keratinocytes isolated from foreskin donors and cultured in serum-free conditions.
- (ii) The current work used a full-thickness skin organoid model ((Keratinocytes seeded on top of donor matched fibroblasts embedded in a fibrin dermal matrix) whereas the previous work was an epidermal equivalent model (Keratinocytes grown in a transwell) .
- (iii) The current work benefited from advancements in proteomic technology and generated a more extensive, detailed proteomic dataset.

The previous work provided, for the first time, evidence that filaggrin deficiency can alter the expression levels of proteins relevant to the pathogenesis of AE, even without an external inflammatory stimulus and the current work supports this.

Mechanistic themes emerging from this study, including inflammatory, proteolytic and cytoskeletal pathways, broadly overlap with findings from this previous work. However, novel findings detected in the current model compared to the prior proteomic analysis (Elias et al., 2017) may be summarised as follows:

- Evidence that filaggrin-deficient keratinocytes are a source of immune signalling.
- Filaggrin deficiency leads to an increase in transcriptional and translational activity but a reduction in multiple markers of terminal differentiation.
- There is a dysregulation of axon guidance (with elements of this pathway represented in both the up- and down-regulated protein sets).

Table 2 presents Reactome data and highlights Axon guidance, and it would be helpful to see this in the context of the other pathways described. I would favour this being included in the PDF, rather than Fig 9, which may be moved to supplementary data.

Thank you for this suggestion. We agree that Table 2 is a valuable summary of the findings from Reactome, but it is too large (including detailed lists of protein IDs, which we are keen to include) to fit into the .pdf. We hope that readers will easily access the full table via the direct link provided to FigShare. We have considered alternative methods to present our Reactome pathway analyses, but the visualisation of pathways within a global over-view does not lend itself to our data where relatively small numbers of pathways (39 and 113) show significant dysregulation.

The volcano plot (Fig 10) is of interest, but more data could be presented here, for example, the 7 points which are log2 fold change >+5 could be identified to make this more informative for the reader. I am uncertain if the colour scheme with p value cut offs is helpful; the authors carefully justify using selected fold change thresholds in the methods section over arbitrary thresholds that are p-value driven and this depiction is at odds with this.

Thank you for this advice. On further consideration we agree that the colour-coding is not helpful and the addition of labels to the most extreme data points is informative for readers. These changes have been made in a corrected version of Fig 10 in the updated manuscript.

Could the title be improved to reflect the more complete nature of this skin equivalent model that incorporates fibroblasts? Also, how were the three elements "increased transcriptional-translational activity, keratinocyte-immune crosstalk and disordered axon guidance" selected or prioritised from the list of Reactome findings?

In response to your helpful suggestion we have added the words 'full thickness' to the title to emphasise the nature of our model. Because the title was already very long we have truncated it, since the main findings ('increased transcriptional-translational activity, keratinocyte-immune crosstalk and disordered axon guidance') are clearly stated in the abstract.

These three elements were prioritised as noteworthy findings based on a combination of GO and pathway analyses as follows:

- (a) Aspects of transcriptional and translational activity are represented in all 10 of the most significant GO terms for Biological process in the consistently down-regulated (Table 1) and up-regulated (Table 3) proteins; 'Metabolism of RNA' is the top Reactome term in the down-regulated protein dataset (Table 2) and the Reactome term 'Translation' is top in the upregulated proteins (Table 4).
- (b) Keratinocyte-immune crosstalk is highlighted to emphasise that these experiments, in which epidermal keratinocytes are isolated from other cells of the immune system, still generate signals classified within the terms 'innate' and 'acquired immunity'. In the Reactome analyses of consistently down-regulated proteins 7/39 enriched pathways are related to the immune or antiviral systems and 15/113 enriched pathways in the up-regulated proteins relate to immune systems or antiviral systems.
- (c) Disordered axon guidance was represented by only one Reactome term in each of the up- and down-regulated protein groups, but together these account for 74/541 (14%) of the proteins in this pathway. This was prioritised as a novel signal of interest, likely to be relevant to AD pathophysiology but not currently targeted for therapy.

There is a minor typographical error on page 3. "Medium was refreshed on alternate day sand the cultures were used for analysis up to day 12 (representing three, five and seven days after lifting to the air-liquid interface)".

Thank you, we have corrected this typo in the updated version of our manuscript.

Competing Interests: No competing interests were disclosed.

Reviewer Report 01 October 2019

<https://doi.org/10.21956/wellcomeopenres.16840.r36427>

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Sabine Werner

Institute for Molecular Health Sciences, Department of Biology, ETH Zurich (Swiss Federal Institute of Technology Zürich), Zürich, Switzerland

The authors generated and characterized 3D organotypic cultures using keratinocytes with filaggrin knock-down or control keratinocytes. Although such cultures had been generated before, they had not been used for proteomics studies. The proteomics analysis provides some insight into the molecular mechanisms underlying the effect of filaggrin on keratinocyte function. The advantage of this system is the focus on the keratinocyte phenotype. The disadvantage is the lack of the influence of other cell types that are important for Atopic Dermatitis pathogenesis. Overall, this is an interesting study, although there is no functional follow-up of any of the differentially expressed proteins. In addition, the statistical analysis requires improvement.

Specific comments:

1. The filaggrin knock-down at the RNA level was only approximately 50%, whereas the knock-down at the protein levels seems more robust. How do the authors explain this inconsistency? Why are there no error bars in Fig. 2 NT-siRNA? The data should be confirmed by immunostaining of the cultures for filaggrin. Staining of the cultures for loricrin would be helpful to confirm abnormalities in the granular layer.
2. It is impossible to see obvious differences in Lucifer yellow penetration and there is also no statistically significant difference. This should be expressed more carefully.
3. The proteomics data provide a very important resource. It would have been helpful to show the validation of some of the data using independent samples and another method, e.g. Western blot and/or immunohistochemistry. This would be easy for selected proteins, such as the different keratins.
4. A comparison of the data with available transcriptomics data from AD patients would be interesting.
5. For the proteomics analyses, it is not clear how the false discovery rate was exactly determined (scrambled or reversed database?) and how false positives were removed. This requires clarification.
6. Statistical analysis to identify proteins with differential abundance between conditions lacks stringency and appears asymmetric with regard to positive and negative effect sizes. Lack of statistically significant hits upon multiple testing correction is a common problem with proteomics datasets. Which multiple testing correction algorithm was applied? Bonferroni or

Benjamini-Hochberg? Did the authors try e.g. a limma moderated t-test that has proven useful for proteomics data? Even without multiple testing correction, a statistical test appears more suitable than selecting differentially abundant proteins only based on consistency between comparisons/experiments. Moreover, the authors apply a cut-off of 2-fold (0.5) to identify proteins with lower abundance upon filaggrin knockdown and of 1.2-fold (1.2) for those with higher abundance in this condition. It appears this skewed effect size has been selected aiming at equalizing hit numbers for pathway enrichment. This should be reconsidered. The terms 'ratio' and 'fold-change' are not used properly, since the authors report actual ratios as fold-changes, e.g. a ratio of 0.5 would correspond to a fold-change of -2 and of 2 to a fold change of +2. Since identifying proteins with statistically significantly differential abundance between conditions is crucial, also for follow-up data analysis, the authors should re-evaluate this part of the study with help of an expert in proteomics statistics.

Is the work clearly and accurately presented and does it cite the current literature?

Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Partly

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Skin Biology, Inflammatory Skin Disease, Wound Healing, Tissue Repair

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 19 Nov 2019

Sara J. Brown, Ninewells Hospital, Dundee, UK

Thank you for your interest in the skin organoid model and our analyses. We believe that a focus on the keratinocyte phenotype is valuable, particularly in AD where the keratinocyte's contribution to barrier function is of particular interest. Whilst we agree that there are many other cell types contributing to AD pathogenesis, we consider that the isolation of keratinocytes may be viewed as an advantage in this experimental system because it provides an opportunity to investigate the specific contribution of keratinocyte biology to features of atopic inflammation and other pathomechanisms in this highly complex trait.

We agree that functional follow-up may be an important step and we have conducted extensive validation in other applications of the skin organoid and a simpler epidermal model.^{1,2} The detailed dataset and our analyses reported here in *Wellcome Open Research* are shared as a resource for the community, to allow others to review the differentially expressed proteins and follow up selected candidates as indicated. We have further clarified this as the principle aim of our publication in Version 2.

Specific comments:

The filaggrin knock-down at the RNA level was only approximately 50%, whereas the knock-down at the protein levels seems more robust. How do the authors explain this inconsistency?

We agree, there was a 0.5-fold reduction in *FLG* mRNA compared to 0.36- and 0.25-fold reduction in pro-filaggrin and filaggrin proteins respectively. These differing levels of reduction in expression reflect the use of siRNA which creates a transient knockdown. siRNA is our preferred methodology because we aim to maintain the primary cells at low passage and to minimise non-physiological experimental influences. The different degrees of knockdown reflect the long experimental time points (10 days post transfection for a mature organoid) and the inherent differences in mRNA and protein stabilities. *FLG* mRNA has a reported half-life of ~2.2 hours³ whereas the half-lives of profilaggrin and filaggrin are approximately 6 and 24 hours respectively.⁴ We have added this additional explanation to the updated manuscript. Since our main experimental aim was to reduce filaggrin at the protein level, we are satisfied that knockdown of the functional monomer is maintained for the duration of our experiment and this degree of reduction is in keeping with the reduction in expression observed in *FLG*-null heterozygotes or atopic skin.

Why are there no error bars in Fig. 2 NT-siRNA?

The filaggrin expression presented in Figure 2 has been calculated in a pair-wise manner (*FLG* -siRNA versus *NT*-siRNA control) for each independent biological donor (N=8) and the replicate data were then combined for statistical analysis. Since only one technical replicate was performed per donor, the *NT*-siRNA condition is calculated as 1 in each biological replicate and hence displays no error in the combined dataset. This internally controlled method of analysis accounts for differences in baseline filaggrin expression and technical variations between replicate experiments.

The data should be confirmed by immunostaining of the cultures for filaggrin.

Thank you for this suggestion. We agree that immunostaining can represent a valuable confirmatory approach, however the H&E stained section show a clear reduction in the granular layer and the filaggrin knockdown at protein level was confirmed by mass spec proteomics (mean 0.27-fold, N=4) which is likely to be more sensitive for the detection of quantitative differences than immunostaining.

Staining of the cultures for loricrin would be helpful to confirm abnormalities in the granular layer.

The electron microscopy (Figure 5) shows the granular layer in some detail, with a clear reduction in the number and size of granules. We therefore did not elect to stain the organoid cultures for loricrin specifically.

It is impossible to see obvious differences in Lucifer yellow penetration and there is also no statistically significant difference. This should be expressed more carefully.

Thank you for bringing this to our attention. We did observe a difference in Lucifer yellow dye penetration in 3/5 of the biological replicates at day 3 but we agree this is not apparent in the mature model at day 7 (example images shown in Figure 8A). We aimed to explain that our quantification (in Figure 8B) shows all of the individual data points and, whilst some experiments showed an increase in dye penetration in the immature organoid, we state “However, this delay in maturation of the ‘outside-to-inside’ barrier function was not consistently demonstrated in all biological replicates.” In light of your comment we have added further clarification to the text.

The proteomics data provide a very important resource. It would have been helpful to show the validation of some of the data using independent samples and another method, e.g. Western blot and/or immunohistochemistry. This would be easy for selected proteins, such as the different keratins.

Thank you, we agree the proteomics data are a valuable resource and we are delighted to share this resource. We also agree it will be relatively easy for other groups to validate proteins of interest in their work; therefore, rather than carrying out validation ourselves for selected keratins (or multiple different proteins as we have for our parallel proteomics analyses, recently published¹) we prefer to share the data for further independent validation.

A comparison of the data with available transcriptomics data from AD patients would be interesting.

We agree that this would be a very interesting analysis; it is not within the remit of this study but we or others may pursue this in the future.

For the proteomics analyses, it is not clear how the false discovery rate was exactly determined (scrambled or reversed database?) and how false positives were removed. This requires clarification.

We apologise for the lack of clarity in this description. The false discovery rate was determined using a reversed database. The peptide and protein false discovery rate was set at 1%, and these identified peptides were denoted with REV_ and removed from further analysis. These details have been added to the revised manuscript.

Statistical analysis to identify proteins with differential abundance between conditions lacks stringency and appears asymmetric with regard to positive and negative effect sizes. Lack of statistically significant hits upon multiple testing correction is a common problem with proteomics datasets. Which multiple testing correction algorithm was applied? Bonferroni or Benjamini-Hochberg? Did the authors try e.g. a limma moderated t-test that has proven useful for proteomics data? Even without multiple testing correction, a statistical test appears more suitable than selecting differentially abundant proteins only based on consistency between comparisons/experiments.

We appreciate that there are no clear guidelines on methodology for the analysis of large proteomics datasets (or indeed many other large datasets) and different expert opinions exist regarding the most appropriate tools and thresholds to define ‘significance’. We have benefited

from the considerable expertise within our multidisciplinary collaboration, leading to the rather simple data analysis that we have performed, whilst providing the full dataset for others to analyse as they may wish.

A full Bonferroni correction does not identify any differentially expressed proteins because of the large number detected in this global analysis. Because of the nature of our data, generated using biological replicate samples (primary cells from 4 separate human donors), and the focus on a common complex trait, we prefer to use the criterion of *consistency* across the biological replicates, with filtering for fold change to provide similar numbers of up- and down- regulated proteins, followed by network and pathway analyses.

Moreover, the authors apply a cut-off of 2-fold (0.5) to identify proteins with lower abundance upon filaggrin knockdown and of 1.2-fold (1.2) for those with higher abundance in this condition. It appears this skewed effect size has been selected aiming at equalizing hit numbers for pathway enrichment. This should be reconsidered.

Thank you for highlighting this question. We have stated clearly and openly that we *did* select these cut-offs aiming to equalize protein numbers in this right-skewed dataset. Using the thresholds of ≤ 0.5 and ≥ 1.2 defines 1422 (~25%) and 1871 (~34%) of the total number of proteins. This is an established approach in proteomic analysis; we appreciate other valid approaches can be applied and we welcome further interrogation of our experimental data. We have further clarified this in the discussion.

The terms 'ratio' and 'fold-change' are not used properly, since the authors report actual ratios as fold-changes, e.g. a ratio of 0.5 would correspond to a fold-change of -2 and of 2 to a fold change of +2.

We apologise for the inconsistency in this terminology and we have corrected this detail throughout the manuscript.

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2. Elias, M.S. *et al.* Proteomic analysis of filaggrin deficiency identifies molecular signatures characteristic of atopic eczema. *J Allergy Clin Immunol* **140**, 1299-1309 (2017).
3. Nirunsuksiri, W., Zhang, S.H. & Fleckman, P. Reduced stability and bi-allelic, coequal expression of profilaggrin mRNA in keratinocytes cultured from subjects with ichthyosis vulgaris. *J Invest Dermatol* **110**, 854-61 (1998).
4. Markova, N.G. *et al.* Profilaggrin is a major epidermal calcium-binding protein. *Mol Cell Biol* **13**, 613-25 (1993).

Competing Interests: No competing interests were disclosed.

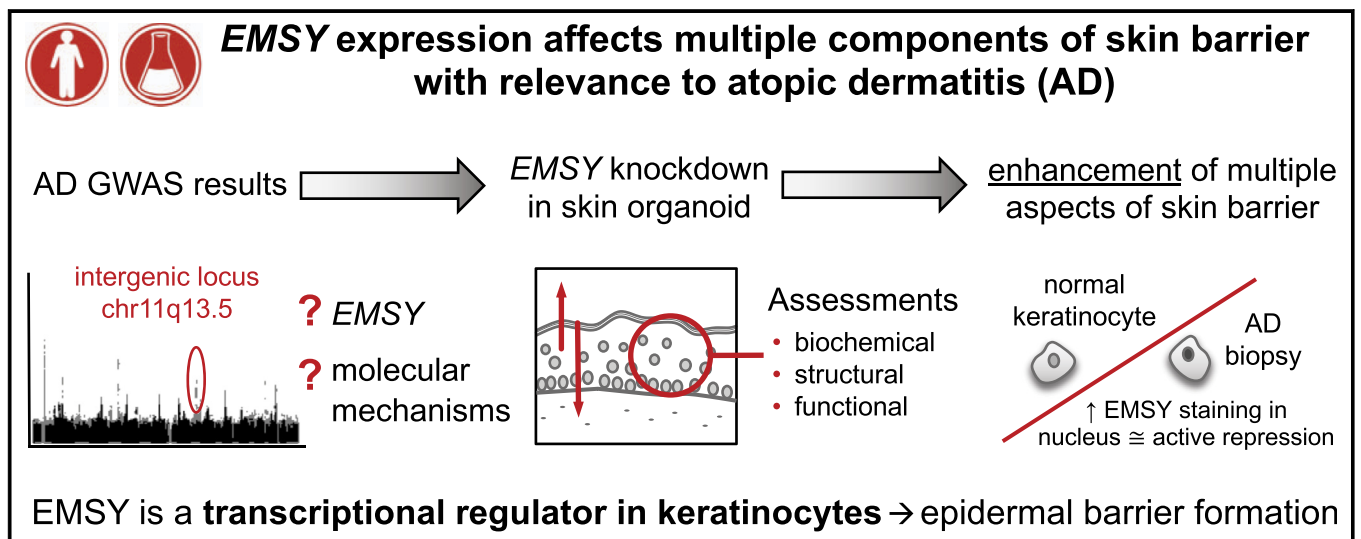
EMSY expression affects multiple components of skin barrier with relevance to atopic dermatitis.

EMSY expression affects multiple components of the skin barrier with relevance to atopic dermatitis



Martina S. Elias, PhD,^a Sheila C. Wright, HNC,^a Judit Remenyi, PhD,^a James C. Abbott, PhD,^b Susan E. Bray, PhD,^c Christian Cole, PhD,^b Sharon Edwards, MBChB,^d Marek Gierlinski, PhD,^b Mateusz Glok,^a John A. McGrath, FRCP,^e William V. Nicholson, PhD,^a Lavinia Paternoster, PhD,^f Alan R. Prescott, PhD,^g Sara Ten Have, PhD,^h Phillip D. Whitfield, PhD,ⁱ Angus I. Lamond, PhD,^h and Sara J. Brown, FRCPE^{a,j} Dundee, Inverness, London, and Bristol, United Kingdom

GRAPHICAL ABSTRACT



Background: Atopic dermatitis (AD) is a common, complex, and highly heritable inflammatory skin disease. Genome-wide association studies offer opportunities to identify molecular targets for drug development. A risk locus on chromosome

11q13.5 lies between 2 candidate genes, *EMSY* and *LRRC32* (leucine-rich repeat-containing 32) but the functional mechanisms affecting risk of AD remain unclear.

From ^athe Skin Research Group, Division of Molecular and Clinical Medicine, School of Medicine, and ^bthe Data Analysis/Bioinformatics Group, ^cthe Dundee Imaging Facility, and ^dthe Centre for Gene Regulation and Expression, School of Life Sciences, University of Dundee; ^eNHS Research Scotland Biorepository Tayside, Ninewells Hospital and Medical School, University of Dundee; ^fthe Department of Pathology, Ninewells Hospital and Medical School, Dundee; ^gSt John's Institute of Dermatology, King's College London (Guy's Campus); ^hthe MRC Integrative Epidemiology Unit, Population Health Sciences, Bristol Medical School, University of Bristol; ⁱthe Lipidomics Research Facility, Division of Biomedical Sciences, University of the Highlands and Islands, Inverness; and ^jthe Department of Dermatology, Ninewells Hospital, Dundee.

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Objectives: We sought to apply a combination of genomic and molecular analytic techniques to investigate which genes are responsible for genetic risk at this locus and to define mechanisms contributing to atopic skin disease.

Methods: We used interrogation of available genomic and chromosome conformation data in keratinocytes, small interfering RNA (siRNA)-mediated knockdown in skin organotypic culture and functional assessment of barrier parameters, mass spectrometric global proteomic analysis and quantitative lipid analysis, electron microscopy of organotypic skin, and immunohistochemistry of human skin samples.

Results: Genomic data indicate active promoters in the genome-wide association study locus and upstream of *EMSY*; *EMSY*, *LRRC32*, and intergenic variants all appear to be within a single topologically associating domain. siRNA-knockdown of *EMSY* in organotypic culture leads to enhanced development of barrier function, reflecting increased expression of structural and functional proteins, including filaggrin and filaggrin-2, as well as long-chain ceramides. Conversely, overexpression of *EMSY* in keratinocytes leads to a reduction in markers of barrier formation. Skin biopsy samples from patients with AD show greater *EMSY* staining in the nucleus, which is consistent with an increased functional effect of this transcriptional control protein.

Conclusion: Our findings demonstrate an important role for *EMSY* in transcriptional regulation and skin barrier formation, supporting *EMSY* inhibition as a therapeutic approach. (J Allergy Clin Immunol 2019;144:470-81.)

Key words: Atopic dermatitis, atopic eczema, *EMSY*, filaggrin, genetics, genomics, organotypic, lipidomics, proteomics, siRNA knockdown

Atopic dermatitis (AD; or eczema¹) is a common inflammatory skin disease with strong heritability.² Genome-wide association studies (GWASs) have identified multiple loci affecting AD risk,³ including effects on the skin barrier and immune function,^{2,4} and it has been demonstrated in other complex traits that molecular mechanisms defined by GWAS loci might represent effective therapeutic targets.⁵

The most widely replicated genetic risk for AD lies within the epidermal differentiation complex on chromosome 1q21.3^{3,6,7}; this includes *FLG*, which encodes the skin barrier protein filaggrin.⁸ Expression levels of filaggrin and its metabolites in the outer epidermis correlate with AD activity.^{9,10} However, this mechanism has not been successfully targeted in therapy development since its discovery more than 10 years ago.

An association peak within an intergenic region on chromosome 11q13.5 was identified in the earliest AD GWAS.⁶ This locus has been replicated in subsequent GWASs¹¹⁻¹³ and meta-GWASs.^{3,7} In addition to AD, the region is associated with multiple atopic phenotypes^{14,15} and other disorders characterized by epithelial barrier dysfunction, including polyallergen sensitization,^{14,16} asthma,¹⁷ allergic rhinitis,¹⁸ food allergy,^{19,20} eosinophil counts,²¹ eosinophilic esophagitis,²² inflammatory bowel disease,²³ and the gut microbiome.²⁴ The AD-associated single nucleotide polymorphisms (SNPs) are in an intergenic region between *LRRC32* (leucine-rich repeat-containing 32), and *EMSY*.

LRRC32 encodes the TGF- β activator LRRC32 (UniProtKB Q14392), previously termed glycoprotein A repetitions predominant (GARP), a membrane protein that binds latent TGF- β 1 on

Abbreviations used

AD:	Atopic dermatitis
DMEM:	Dulbecco modified Eagle medium
EGF:	Epidermal growth factor
<i>FLG</i> :	Gene encoding filaggrin
GO:	Gene ontology
GWAS:	Genome-wide association study
Hi-C:	Genome-wide chromosome conformation capture and high-throughput sequencing to identify regions of DNA showing interaction in 3-dimensional space
<i>LRRC32</i> :	Leucine-rich repeat-containing 32 gene, encoding the glycoprotein A repetitions predominant (GARP) protein
NHK:	Normal human keratinocytes
NDF:	Normal dermal fibroblasts
qPCR:	Quantitative PCR
siRNA:	Small interfering RNA
SNP:	Single nucleotide polymorphism
TEWL:	Transepidermal water loss

the surfaces of activated regulatory T cells.²⁵ *LRRC32* has been proposed as a causal gene for atopic skin inflammation,²⁶ but the credible SNPs identified by GWASs at this locus are all intergenic,³ suggesting that regulatory rather than coding variants drive the association.

EMSY, also known as BRCA2-interacting transcriptional repressor and previously termed *C11orf30*, codes for the protein *EMSY* (UniProtKB Q7Z589), which is expressed in multiple human tissues, including cerebellum, breast, lung, ovary, uterus, and skin (GTEx RNA-seq, V7). *EMSY* has been characterized as a transcriptional regulator, either repressing transcription as part of a chromatin remodeling complex or activating transcription as part of a histone H3-specific methyltransferase complex.²⁷ *EMSY* amplification is associated with DNA damage and increased risk of malignancy in breast and ovarian tissue.²⁷ *EMSY* can also play a role in inflammation because the protein kinase AKT1 regulates the interferon response through phosphorylation of *EMSY*,²⁸ but its role in skin remains undefined.

We set out to investigate, using genetic and genomic data, whether *EMSY*, *LRRC32*, or both, showed evidence of activity in human skin cells. We then investigated the mechanism of effect using functional and multi-omics analysis of organotypic skin, followed by immunostaining of AD biopsy samples.

METHODS

Human tissues and cells

All human tissues were obtained with written informed consent from donors under the governance of and with ethical approval from the NHS Research Scotland Biorepository in Tayside. Primary keratinocytes and donor-matched primary fibroblasts were extracted from skin samples discarded from plastic surgery procedures. AD skin samples were identified from the hospital pathology database as consecutive unselected cases. Demographic details are provided in the [Methods](#) section in this article's Online Repository at www.jacionline.org.

Cell and skin organotypic cultures

Normal human keratinocytes (NHKs) and normal dermal fibroblasts (NDFs) were isolated from healthy breast skin by means of sequential collagenase D and trypsin EDTA digestion, as previously described.²⁹ NHKs

were cocultured in RM medium (3:1 Dulbecco modified Eagle medium [DMEM]/Hams F12, 10% FCS, 0.4 $\mu\text{g/mL}$ hydrocortisone, 5 $\mu\text{g/mL}$ insulin, 10 ng/mL epidermal growth factor [EGF], 5 $\mu\text{g/mL}$ transferrin, 8.4 ng/mL cholera toxin, and 13 ng/mL liothyronine; Sigma-Aldrich, Gillingham, Dorset, United Kingdom) along with mitomycin C-inactivated 3T3 feeder cells.³⁰ EGF was omitted for the first day of culture. NDFs were cultured in DMEM supplemented with 10% FCS under standard conditions.

Fibrin gel dermal equivalents were prepared by using a protocol adapted from published methods.³¹⁻³³ A volume of 0.5 mL fibrinogen (35 mg/mL in NaCl; Sigma-Aldrich) and 0.5 mL of thrombin (3 U/mL in 2 mmol/L CaCl_2 /1.1% NaCl; Sigma-Aldrich) were combined on ice, supplemented with 200,000 NDFs and aprotinin (0.1 U/mL; Sigma-Aldrich), and transferred to a 12-well plate. After 30 minutes of incubation at 37°C, the gels were covered in medium (DMEM, 10% FCS, and 0.1 U/mL aprotinin) and cultured overnight (day 1). The following day, medium was replaced with RM medium excluding EGF, 0.1 U/mL aprotinin, and 2×10^6 suspended NHKs (day 2). This was refreshed daily for the next 2 days (day 3 and 4) with RM medium containing 0.1 ng/mL EGF and 0.1 U/mL aprotinin. On day 5, gels were carefully removed from wells by using a plastic spatula and lifted onto custom-made steel grids lined with nylon gauze (Millipore, Livingston, United Kingdom). RM medium supplemented with 0.1 ng/mL EGF and 0.1 U/mL aprotinin was added up to the base of the grid, enabling the fibrin gels to be nourished from below and the epidermis cultured at the air-liquid interface. Medium was refreshed every other day until day 12 when the cultures were analyzed. Where required, the epidermis was isolated from fibrin gel after hypertonic saline-induced split (4 hours, 1 mol/L NaCl, 4°C).

Small interfering RNA-mediated knockdown

NHKs were reverse transfected immediately before inclusion in the organotypic cultures by using RNAiMax transfection reagent (Life Technologies, Carlsbad, Calif), according to the manufacturer's instructions. Briefly, small interfering RNA (siRNA) complexes were formed in Opti-MEM medium (20 $\mu\text{mol/L}$ siRNA and 5 μL of RNAiMAX) and, after 20 minutes of incubation, combined with 2×10^6 suspended NHKs and transferred to the preprepared dermal substrate. A pool of 4 siRNA duplexes was used (EMSY: LQ-004081-00-0002, FLG: LQ-021718-00-0002, control: ON-TARGETplus nontargeting siRNA #4 D.001810-04-20; Dharmacon, Lafayette, Colo).

EMSY overexpression in primary keratinocytes

A second-generation lentiviral system was used, as follows. Pseudoviral particles were prepared with psPAX2 packaging (catalog no. 12260; AddGene, Watertown, Mass) and pMD2.G plasmid (catalog no. 12259; AddGene), which were cotransfected with the control pLenti-C-mGFP-P2A-puromycin-tagged cloning vector (catalog no. PS100093; OriGene, Rockville, Md) plasmid or with the Lenti-ORF clone of mGFP-tagged-human chromosome 11 open reading frame 30 (*C11orf30*; catalog no. RC216916L4; OriGene) plasmid with Lipofectamine-3000 transfection reagent (catalog no. L3000008; Invitrogen, Carlsbad, Calif), according to the manufacturer's protocol, into 293T packaging cells for 16 hours. The next morning, the cells were washed twice with PBS to remove excess plasmid DNA, and the medium was replaced with virus-producing medium (20% FBS/DMEM). Forty-eight, 72, and 96 hours after transfection, the first, second, and third viral supernatants were harvested. Viral supernatants were spun down at 1200 rpm for 15 minutes and filtered with a 0.45- μm filter.

Primary human keratinocytes from donor skin were transduced twice. The first transduction was in RM medium without EGF. After treatment, the keratinocytes with trypsin and 1×10^6 cells/well were mixed with 10 $\mu\text{g/mL}$ Polybrene (hexadimethrine bromide; catalog no. H9268; Sigma-Aldrich) and 1 or 2 mL of viral supernatant and then plated onto 6-well plates and cultured overnight. The second transduction was performed in monolayer culture using RM medium without EGF, 10 $\mu\text{g/mL}$ Polybrene, and 1 or 2 mL of viral supernatant. Cells were incubated for 90 minutes at 37°C, followed by centrifugation at 1200 rpm. To eliminate excess virus, cells were

washed twice with PBS, and the medium was replaced with RM media (3:1 DMEM/Hams F12, 10% FCS, 0.4 $\mu\text{g/mL}$ hydrocortisone, 5 $\mu\text{g/mL}$ insulin, 10 ng/mL EGF, 5 $\mu\text{g/mL}$ transferrin, 8.4 ng/mL cholera toxin, and 13 ng/mL liothyronine). Fresh RM medium was replaced every second day, and samples were harvested on day 10 after transduction as a differentiated culture.

Fluorescent dye penetration

Fifty microliters of 1 mmol/L Lucifer yellow dye (Sigma-Aldrich) was added to the epidermal surface of the organotypic culture and incubated at 37°C for 4 hours. Metal cloning rings were used to control uniform dosing on the epidermal surface. Lucifer yellow was removed, and the organotypic cultures were washed in PBS before formalin-fixed paraffin embedding under standard conditions. Four-micrometer sections were deparaffinized, counterstained with 4'-6-diamidino-2-phenylindole dihydrochloride (1 $\mu\text{g/mL}$ for 10 minutes; Life Technologies, Carlsbad, Calif), and imaged with a confocal Zeiss LSM710 microscope (Zeiss, Oberkochen, Germany). Quantification of dye penetration in the upper dermis (average intensity in the upper 40 μm) was performed with Zeiss Zen software and compared by using paired *t* tests.

Transepidermal water loss

Organotypic cultures were equilibrated at room temperature and atmospheric conditions for 30 minutes before transepidermal water loss (TEWL) was measured at 2 locations on the epidermal surface with an AquaFlux AF200 instrument (Biox Systems, London, United Kingdom) with a custom (5 mm in diameter) probe head. TEWL measurements were taken every second for a minimum of 60 seconds until a stable reading, as determined by using the software, was obtained.

Capacitance

Organotypic cultures were equilibrated at room temperature and atmospheric conditions for 30 minutes before measurement of epidermal surface capacitance as a measure of water content with a Corneometer (Courage and Khazaka, Cologne, Germany). Three measurements were recorded from each organotypic culture, and the mean was calculated.

Protein data analysis

Network analysis was performed by using Ingenuity Pathway Analysis (Qiagen Ingenuity, version 01-12; Qiagen, Hilden, Germany). Gene ontology (GO) enrichment analysis was performed in the Gene Ontology Consortium online tool by using PANTHER classification <http://www.geneontology.org/page/go-enrichment-analysis> (accessed August 28, 2018). A volcano plot was generated in R/ggplot2 by using human proteins detected in all 4 of the replicates to calculate the fold change (nontargeting control/EMSY knock-down) for each donor with \log_{10} transformation and a *t* test for significance.

Lipid staining

Frozen sections of skin organotypic samples were cut and air-dried onto slides before formalin fixation and rinsing with 60% isopropanol. Oil Red O (Sigma-Aldrich) working solution was freshly prepared, and sections were stained for 15 minutes, rinsed with 60% isopropanol, and then lightly counterstained with alum hematoxylin before a final rinse with distilled water.

Additional Methods are described in this article's Online Repository at www.jacionline.org.

RESULTS

Genomic data support EMSY and LRRC32 as candidate genes in skin

The AD-associated SNPs at the chromosome 11q13.5 locus are approximately 27 kb downstream of *EMSY* (Human Genome

UCSC Genome Browser Human and Ensembl (GRCh38/hg38)

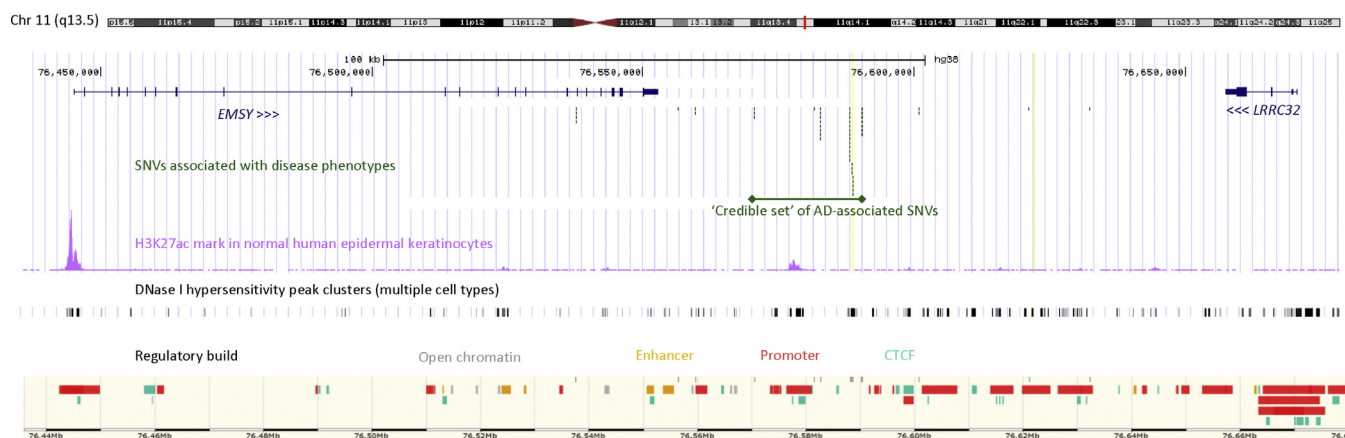


FIG 1. Regulatory features of the AD risk locus and adjacent genes on chromosome 11q13.5. Genes (blue) flanking the locus with multiple disease-associated SNPs (green) were identified by using a GWAS. *EMSY* has a protein-coding region spanning 106 kb, with 20 exons producing 18 splice variants. *LRRC32* spans 13 kb, including 3 exons. The location of the credible set of AD-associated SNPs³ is marked. H3K27ac marks (pink) and DNase I hypersensitivity data (gray) are from the Encyclopedia of DNA Elements (ENCODE; accessed June 2018). Regulatory features in the same region of chromosome 11 are from Ensembl (GRCh38.12, accessed April 2019).

Nomenclature Committee: 18071, ENSG00000158636) and approximately 77 kb downstream of *LRRC32* (Human Genome Nomenclature Committee: 137207, ENSG00000137507). The credible set identified by mapping this locus lies entirely within an intergenic region (Fig 1). Encyclopedia of DNA Elements (ENCODE) and Ensembl data predict multiple regulatory features within the region of disease risk SNPs, and there are putative promoters upstream of each gene (Fig 1). Focusing on skin, there are histone H3K27ac marks indicating active enhancers or promoters in NHKs in the AD risk locus and at the 3' end of *EMSY* but not *LRRC32* (Fig 1).

Enhancer-promoter interactions can occur by proximity in 3-dimensional space³⁴ and show cell lineage specificity³⁵; therefore we reanalyzed 2 sets of genome-wide chromosome conformation capture and high-throughput sequencing (Hi-C) data from NHKs, to identify regions of DNA showing interaction in 3-dimensional space. Interrogation of Hi-C data³⁶ shows that the intergenic SNPs, as well as *EMSY* and *LRRC32* all lie within a single topologically associated domain in keratinocytes (see Fig E1 in this article's Online Repository at www.jacionline.org), supporting a possible functional interaction. Analysis of promoter-capture Hi-C³⁷ in differentiating keratinocytes showed evidence of interaction between the promoter region of *LRRC32* and the intergenic SNP locus, but these data were not sufficiently detailed to determine whether *EMSY* also shows conformational interaction (see Fig E1).

Gene expression data³⁸ and our own single-molecule RNA-sequencing analysis³⁹ confirm expression of each gene in the skin, but there is no significant difference in *EMSY* or *LRRC32* mRNA abundance in atopic skin compared with nonatopic control skin ($P > .05$, see Fig E2 in this article's Online Repository at www.jacionline.org). However, *EMSY* is more highly expressed in skin than *LRRC32* at the protein level (<https://www.proteinatlas.org/>), and it has not previously been studied in keratinocyte biology; therefore *EMSY* was selected for further detailed investigation.

EMSY knockdown in a skin organotypic model enhances barrier function

To investigate a functional effect of *EMSY* in skin, we used primary human keratinocytes seeded onto a dermal equivalent, which forms an organotypic model with stratified layers that effectively recapitulate the structure and gene expression patterns of human skin.⁴⁰ The model also demonstrates functional parameters controlling the entry and exit of small molecules, and this can be used to quantify effects on barrier formation and function.⁴¹ siRNA knockdown of *EMSY* expression was achieved by means of transfection of keratinocytes immediately before seeding onto the dermal equivalent. Knockdown was confirmed at the mRNA and protein levels, persisting to 10 days in organotypic culture (Fig 2, A-C). Equivalent effects were seen by using individual and pooled siRNAs (see Fig E3 in this article's Online Repository at www.jacionline.org). *EMSY* knockdown produced a marked phenotypic change (Fig 2, D), including thickening of the epidermal cell layer and stratum corneum (Fig 2, E). There was an increase in the number of layers within the stratum corneum and the frequency of corneodesmosomes (Fig 2, F). The stratum granulosum, the site of filaggrin expression,⁸ was also more prominent (Fig 2, D), and increased filaggrin expression was confirmed by using quantitative PCR (qPCR; $n = 7$ replicates, mean \pm SEM fold change = 2.00 ± 0.41 compared with the nontargeting control) and Western blotting ($n = 7$, mean fold change = 1.97 ± 0.22 ; Fig 2, G).

In our skin culture model stratum corneum hydration, TEWL, and Lucifer yellow dye penetration progressively decrease as the skin barrier is formed (see Fig E4 in this article's Online Repository at www.jacionline.org). *EMSY* knockdown in the skin model resulted in a reduction in stratum corneum hydration (Fig 3, A), a reduction in TEWL (Fig 3, B), and a reduction in penetration of the Lucifer yellow dye (Fig 3, C and D), which is in keeping with enhanced and accelerated barrier development compared with control siRNA treatment.

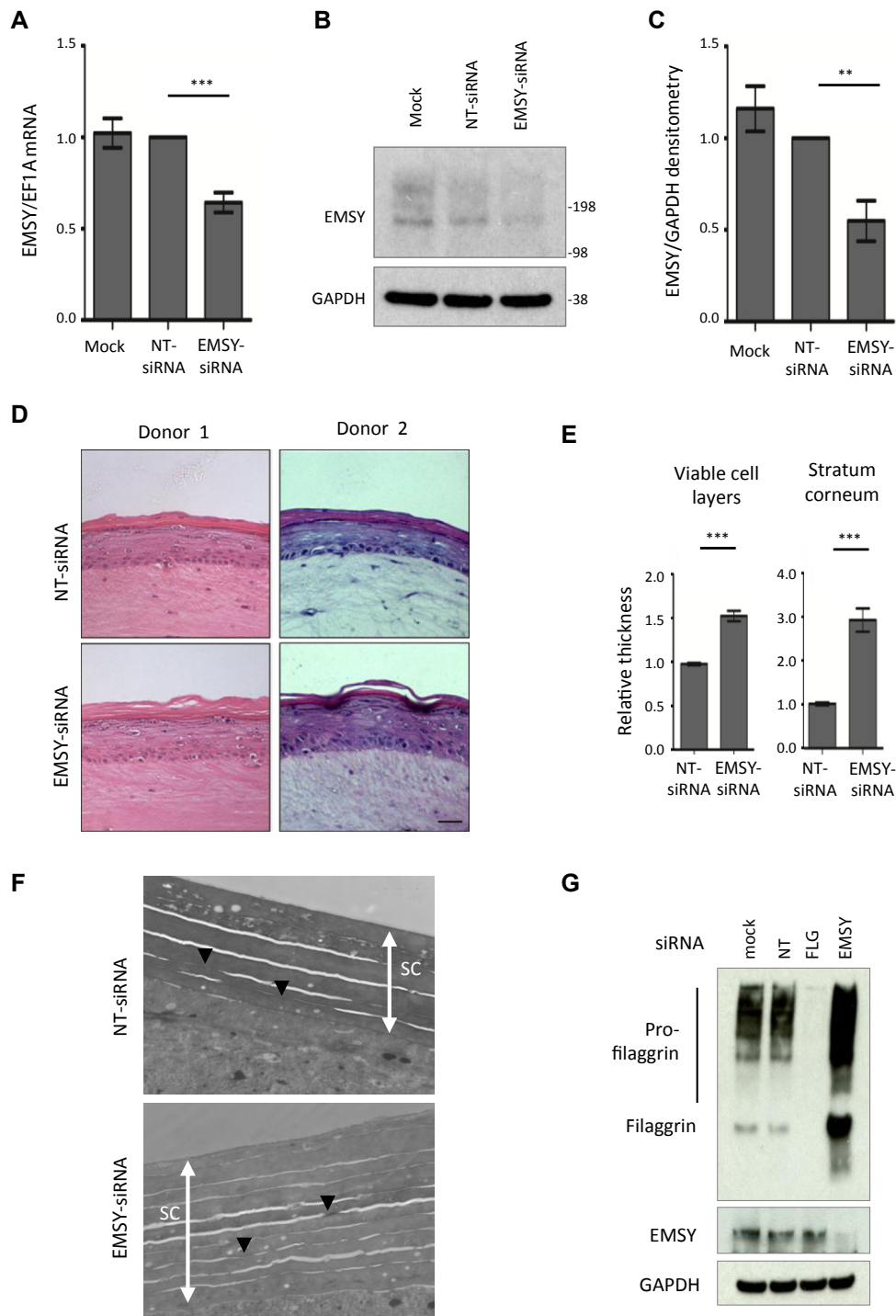


FIG 2. Biochemical and histologic effects of *EMSY* knockdown in a skin organotypic model *in vitro*. **A**, qPCR showing knockdown of *EMSY* mRNA after 10 days in culture (7 days after lifting to the air-liquid interface) normalized to a nontargeting control-treated sample ($n = 7$, mean 36% reduction compared with the nontargeting control). **B** and **C**, Western blot showing knockdown of *EMSY* protein after 10 days in culture (Fig 2, **B**) and densitometry to quantify protein knockdown normalized to a nontargeting control (Fig 2, **C**; $n = 7$). **D**, Appearance of organotypic cultures showing an increased granular layer and thickened stratum corneum in response to *EMSY* siRNA knockdown; representative images were replicated in 10 independent donor experiments. Scale bar = 25 μ m. **E**, Relative thickness of epidermal layers ($n = 10$). **F**, Transmission electron microscopy of stratum corneum showing thickness of the stratum corneum (white arrows) with an increase in the number of layers and number of corneodesmosomes (black arrowheads). **G**, Western blot showing filaggrin expression in skin organotypic cultures untreated (mock) and treated with nontargeting siRNA, *FLG* siRNA, and *EMSY* siRNA 7 days after lifting to the air-liquid interface. $**P < .001$ and $***P < .0001$, paired *t* test. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *NT*, nontargeting control siRNA. Bars show means and SEMs.

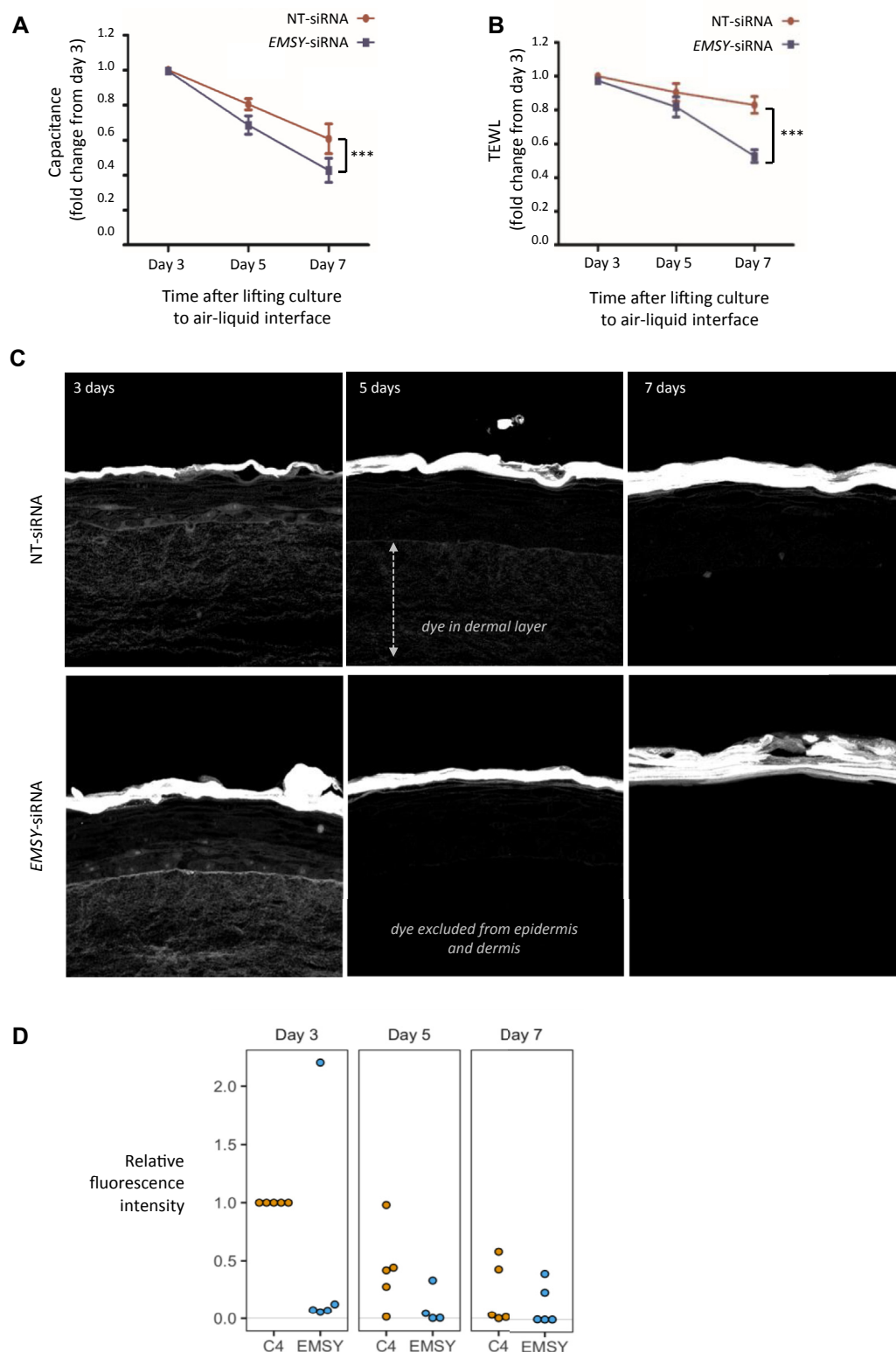


FIG 3. Functional effects of *EMSY* knockdown in the skin organotypic model. **A**, Water content of the stratum corneum measured by capacitance on the skin surface ($n = 5$). *** $P < .0001$, paired t test. **B**, Water evaporation from the epidermal surface measured as TEWL ($n = 5$). **C**, Visualization of fluorescent dye penetration through the epidermis and dermis after 4 hours in skin organotypic cultures developed for 3, 5, and 7 days after lifting to the air-liquid interface. Representative images are from experiments replicated in 5 independent donor experiments. **D**, Overexpression of *EMSY* in primary human keratinocytes and concomitant reduction in expression of differentiation markers ($n = 5$).

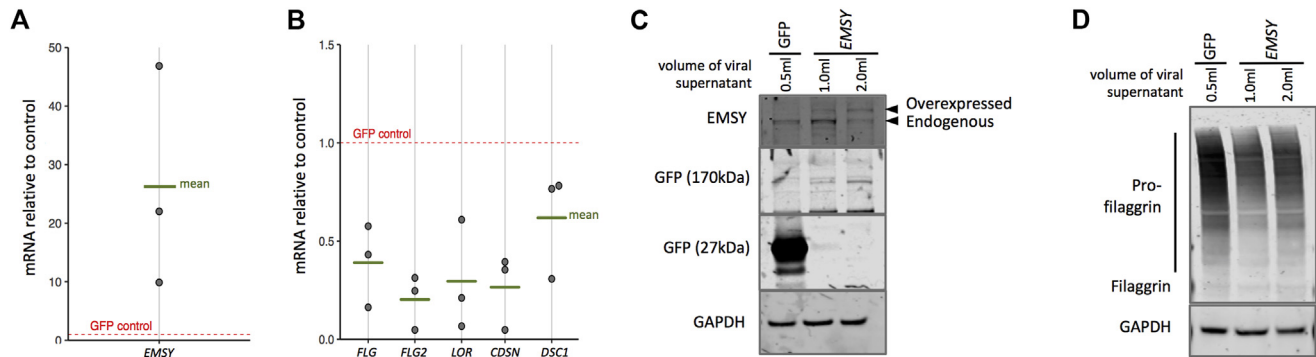


FIG 4. Functional effects of *EMSY* knockdown in a skin organotypic model. Overexpression of *EMSY* in primary human keratinocytes (**A**) and concomitant reduction in expression of differentiation markers at the mRNA (**B**) and protein (**C** and **D**) levels ($n = 3$). Quantification of protein blots by using densitometry is shown in Fig E5. *GAPDH*, Glyceraldehyde-3-phosphate dehydrogenase; *GFP*, green fluorescent protein.

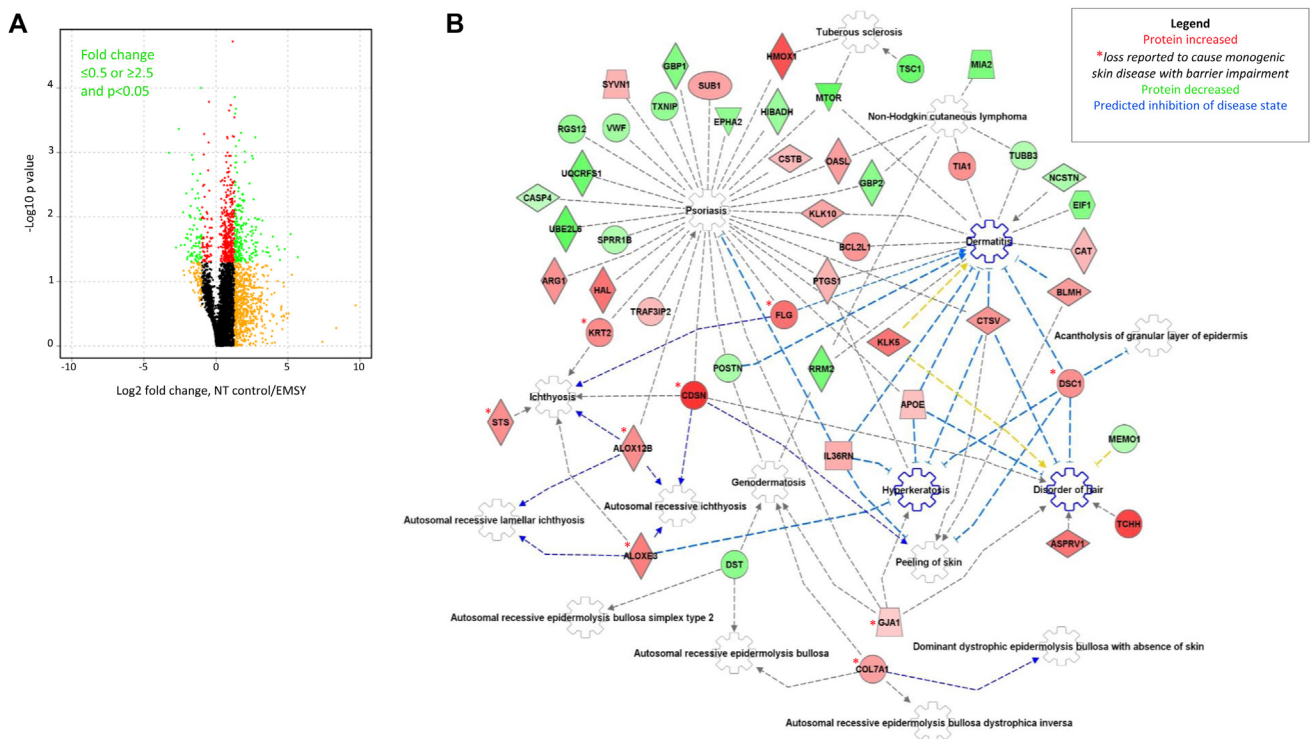


FIG 5. Proteomic analysis of *EMSY* knockdown in a skin organotypic model. **A**, Volcano plot showing mean fold change in 4 biological replicate samples comparing the nontargeting (NT) control siRNA-treated model with *EMSY* siRNA knockdown. t Test results are color coded red ($P < .05$), orange (fold change ≥ 2.5 or ≤ 0.5), or green ($P < .05$ and fold change ≥ 2.5 or ≤ 0.5). **B**, Ingenuity Pathway Analysis (Qiagen) of proteins consistently upregulated or downregulated ($0.5 \geq \text{fold change} \geq 2.5$) in 3 or more of 4 biological replicates, showing proteins defective in monogenic skin diseases with similarities to AD, including ichthyoses, hyperkeratosis, or skin fragility (red asterisks) and an enhancement of pathways predicted to inhibit the development of dermatitis, hyperkeratosis, and hair disorders (blue cogwheels).

EMSY overexpression reduces filaggrin expression

In contrast to the phenotype observed with *EMSY* knockdown, overexpression of *EMSY* in primary human keratinocytes resulted in a reduction in multiple markers of differentiation and barrier formation at the mRNA and protein levels (Fig 4 and see Fig E5 in this article's Online Repository at www.jacionline.org).

Proteomic analysis reveals pathways inhibiting the development of dermatitis

To assess in more detail the *EMSY* knockdown phenotype, we applied tandem mass spectrometric global proteomic analysis (MS/MS) to donor-matched control and *EMSY* knockdown organotypic experiments from 4 independent donors. Total epidermal protein extracts were fractionated by using high-pH

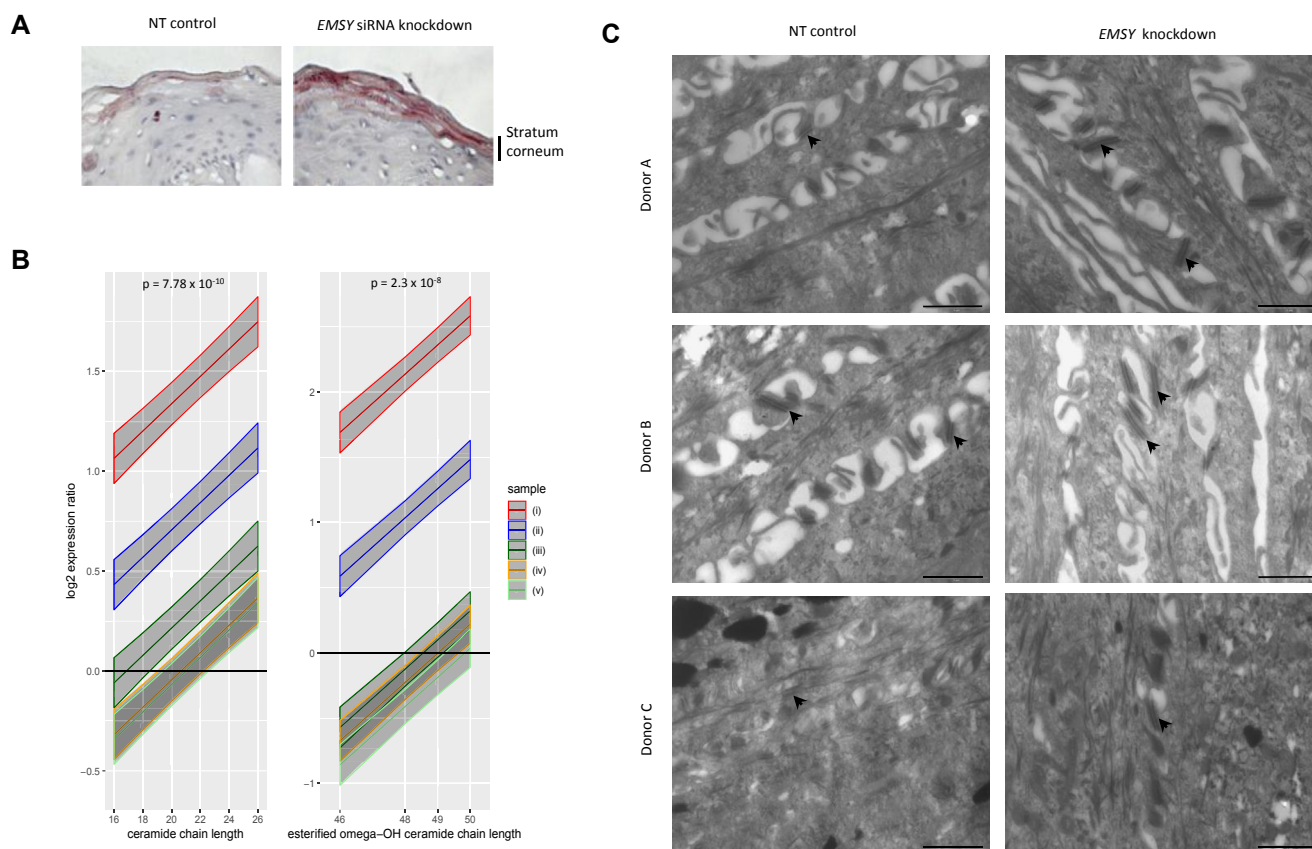


FIG 6. Lipid and ultrastructural analyses of EMSY knockdown in the skin organotypic model. **A**, Lipid staining of skin organotypic samples treated with nontargeting (NT) siRNA control and EMSY siRNA and Oil Red O stain neutral lipids. **B**, Mass spectrometric lipid analysis showing a greater abundance of ceramides with longer chain length in EMSY siRNA-treated organotypic skin compared with a nontargeting siRNA control. The x-axis shows nonhydroxy fatty acid carbon chain length ($n = 5$ biological replicate samples). Lines show the best fit of the linear model and 95% CIs (shaded areas). P values are for the term in the linear model for lipid chain length. **C**, Transmission electron microscopy: osmium tetroxide postfixed and stained, showing increased size of desmosomal structures (arrows) in the EMSY knockdown skin model. Scale bar = 1 μ m.

reversed-phase chromatography before tandem mass spectrometry, which identified more than 9000 proteins per sample (see Fig E6 in this article's Online Repository at www.jacionline.org). qPCR and Western blotting confirmed knockdown of EMSY in each sample. A greater proportion of proteins showed increased rather than decreased expression on EMSY knockdown (Fig 5, A), indicating that EMSY's predominant role is as a transcriptional repressor in this tissue.

Data were filtered for proteins showing changes in the same direction consistently across all 4 biological replicates and fold changes of 2.5 or greater ($n = 154$ proteins) or 0.5 or less ($n = 130$ proteins) in at least 3 of 4 of the biological replicates. Consistent direction of change was used as a criterion to focus analysis on changes of relevance to EMSY knockdown and showing reproducibility across different donors to minimize any effect of interindividual differences.

GO analysis of the upregulated proteins showed enrichment for the biological processes termed establishment of the skin barrier (GO: 0061436), skin development (GO: 0043588), regulation of water loss through the skin (GO: 0033561), and cornification (GO: 0070268; each false discovery rate: $P \leq .037$). GO analysis of the downregulated proteins showed enrichment of cellular

components termed cytosol (GO: 0005829), the mitochondrial membrane (GO: 0031966), and the Golgi membrane (GO: 0000139; each false discovery rate: $P \leq .010$). The full lists of GO terms defined by using this analysis are shown in Fig E7 in this article's Online Repository at www.jacionline.org.

Pathway analysis (Ingenuity, version 01-12; Qiagen) identified upregulation of pathways predicted to inhibit the development of dermatitis and ichthyosis (Fig 5, B).⁴²⁻⁴⁸ Differential expression levels of proteins from these pathways were tested for validation by using qPCR, Western blotting, and/or immunostaining of organotypic skin. All positive and negative findings and untested proteins are displayed in Fig E8 in this article's Online Repository at www.jacionline.org.

EMSY knockdown increases long-chain ceramides

Organotypic samples showed an increase in abundance of epidermal lipid staining with EMSY knockdown (Fig 6, A), which is in keeping with the increase in levels of proteins involved with lipid metabolism (eg, STS, ALOXE3, ALOX12B, and APOE). Mass spectrometric lipid analysis showed an increase in long-chain ceramides and esterified omega-hydroxy-ceramides species

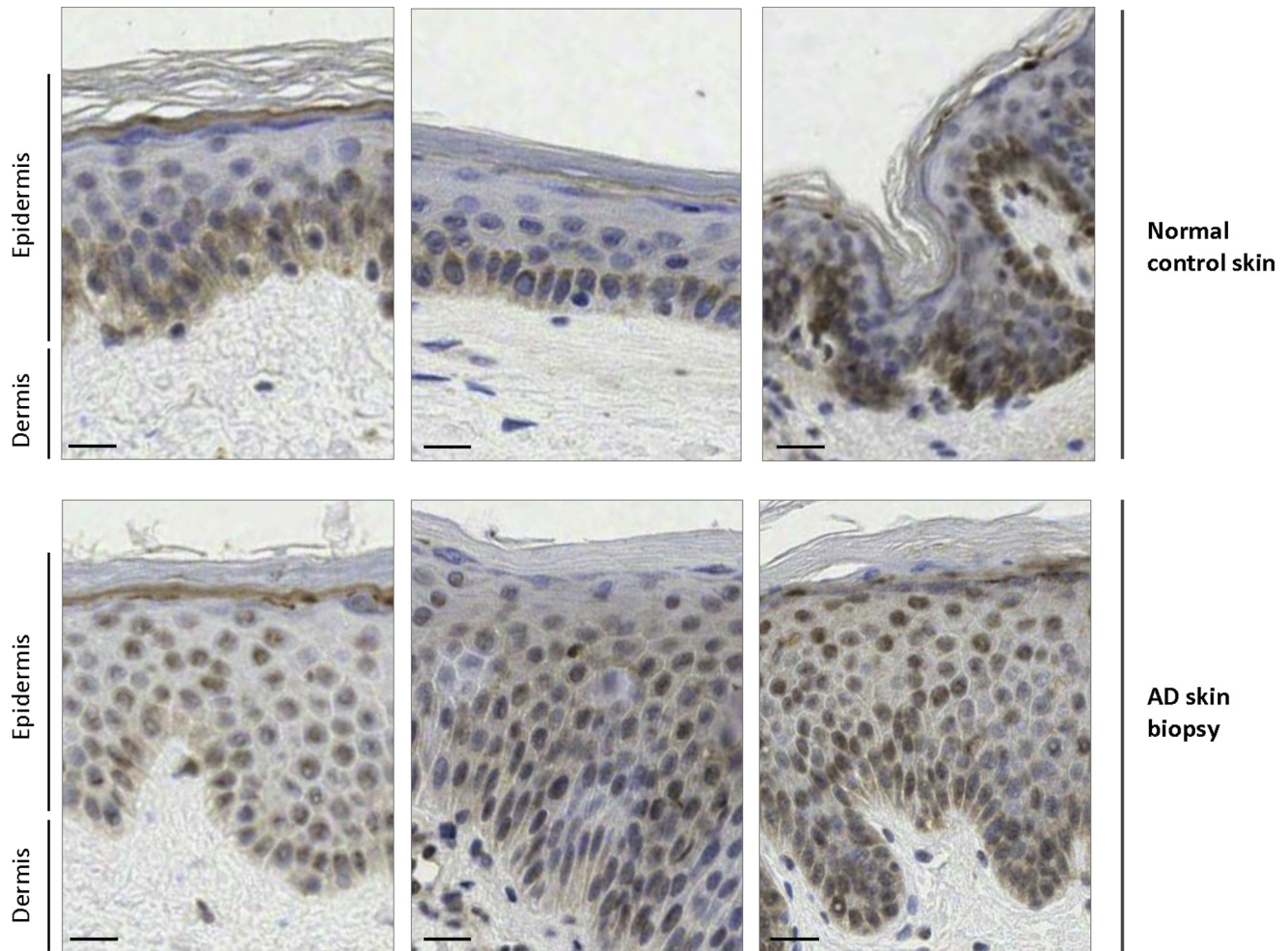


FIG 7. EMSY localization in skin of patients with and without AD. Immunohistochemistry showing EMSY staining in a predominantly basal distribution in normal human skin in contrast to nuclear staining extending throughout the epidermis in AD lesions. Representative images are taken from a tiled image of the whole specimen viewed by using Deep Zoom. Scale bar = approximately 20 μ m.

in organotypic samples with *EMSY* knockdown (Fig 6, B); these are the ceramides that have previously been reported to show a reduction in AD skin samples.⁴⁹

EMSY knockdown increases cell-cell adhesion structures

Ultrastructural analysis demonstrated an increase in desmosome size (Fig 6, C), which is consistent with the observed increase in desmocollin 1 levels. An increase in the number of layers within the stratum corneum was also observed, which is consistent with the increase in corneodesmosin levels (Fig 2, F, and see Fig E9 in this article's Online Repository at www.jacionline.org).

EMSY shows predominantly nuclear localization in the skin affected by AD

To investigate *EMSY* expression in human skin samples, we compared 18 normal control skin samples and biopsy specimens from 14 patients with spongiotic dermatitis, AD, or both. Immunohistochemistry showed considerable interindividual variability

in *EMSY* staining, despite careful standardization of staining conditions, and there was no consistent difference in total intensity between cases and control subjects (see Fig E10 in this article's Online Repository at www.jacionline.org). However, all 14 AD cases showed greater nuclear than cytoplasmic staining, and 12 (86%) of 14 cases showed predominantly nuclear staining extending throughout the epidermis compared with 2 (11%) of 18 control subjects (example images are shown in Fig 7, and all images are shown in Fig E10). Similarly, control organotypic samples showed *EMSY* staining in nuclei throughout the epidermis, whereas the organotypic cultures with *EMSY* knockdown showed expression predominantly in the basal epidermis in keratinocyte nuclei. Thus increased nuclear expression is consistent with increased activity of *EMSY* as a transcriptional regulator within keratinocytes in lesional skin of patients with AD.

DISCUSSION

GWASs have provided valuable insight into the pathogenic mechanisms of many common complex traits, and some have been exploited for targeted therapy development.^{5,50,51} However, many of the loci identified by GWASs are intergenic, and

the molecular mechanisms require detailed functional analysis performed in disease-relevant tissue to characterize important pathomechanisms.⁵² Increasing understanding of the regulatory features in intergenic DNA^{53,54} provides insight into possible effector genes, either locally or distant from variants identified by GWAS. Skin as an organ that can be cultured *in vitro* offers an opportunity to investigate genetic mechanisms using primary cells, quantification of barrier function, and detailed molecular analyses. Cells extracted from normal human skin have been used in our model to investigate the effect of a single candidate gene without the multiple genetic and epigenetic effects that would be coinherited in cells harvested from a patient with AD.² Use of primary cells more closely represents skin physiology than an immortalized cell line, and replication in multiple donors is used to control for inter-individual variation.

Our findings from genomic data and *in vitro* and *ex vivo* analyses converge to provide an understanding of the role of EMSY in skin barrier function. The epigenetic regulatory mechanisms indicated by methylation and chromosomal confirmation (Fig 1) provide a possible mechanism by which the intergenic SNPs associated with AD can affect EMSY expression. However, additional epigenetic mechanisms,⁵⁵ including other forms of histone modification, micro-RNAs, and long noncoding RNAs, might also play roles in EMSY transcriptional control. The greater nuclear localization of EMSY in lesional skin of patients with AD indicates increased activity of this transcriptional repressor. This is in keeping with our findings in primary cell cultures, where EMSY overexpression leads to a reduction in levels of multiple proteins that have previously been shown to be biomarkers for AD.⁵⁶⁻⁵⁸ Improvements in analytic capacity have increased understanding of the importance of lipid composition in epidermal physiology and pathophysiology, including atopic disease,⁵⁹ and the observed increase in relevant lipid species emphasizes the role of EMSY in controlling multiple aspects of skin barrier function.

It is interesting to note that EMSY knockdown increases filaggrin expression, whereas EMSY overexpression leads to a marked reduction in profilaggrin levels. This might in part explain the observation that the genetic risk variants in chromosome 11q13.5 and *FLG* show a multiplicative effect in population analysis.⁶⁰ Chromosome 11q13.5 and the *FLG* locus also both show their strongest associations within a subgroup of early-onset and persistent AD in childhood,⁶¹ which is in keeping with their combined effect leading to a more severe phenotype.

EMSY loss-of-function mutations are rare: they are detected in 1/60,000 unrelated subjects in the Exome Aggregation Consortium,⁶² the minor allele frequency is approximately 0.001 in the Exome Variant Server,⁶³ and there are no homozygous loss-of-function genotypes identified in gnomAD.⁶⁴ This is consistent with our finding that even a modest reduction in expression (mean approximately 33% reduction in mRNA or protein in our experimental model) results in a marked phenotypic change in organotypic cultures.

Together, these observations indicate that loss-of-function mutations are likely to have a deleterious effect, and more subtle modulation of EMSY expression is required for optimal skin barrier function. Therefore it is tempting to speculate that the control of *FLG* expression and skin barrier function through EMSY might be a more sensitive mechanism to exploit than targeting filaggrin directly. It might also be a

more tractable target because the therapeutic effect would be achieved by knockdown of EMSY rather than attempting to increase expression of filaggrin.

Loci reaching statistical significance in genome-wide analyses might reflect the combination of more than 1 functional association, and multieffect loci have been observed in patients with AD, as well as other complex traits.^{12,65} Our finding of a role for EMSY in keratinocytes does not preclude an additional effect of EMSY through expression in T cells,⁶⁶ which are known to play a key role in AD pathogenesis.² Intriguingly, immunohistochemistry also reveals EMSY staining within the nuclei of cells in the dermis (Fig 6 and see Fig E10). This might represent an inflammatory infiltrate, including T cells, and warrants further investigation. Similarly, the genomic promoter-capture Hi-C analysis (see Fig E1) provides more support for a possible promoter-promoter interaction between the GWAS locus and *LRRC32* than EMSY, suggesting that an effect through *LRRC32* expression in keratinocytes or T cells might also play a role in the pathogenesis of AD. Further work, including high-resolution 3C,⁶⁷ could be used to finely map these important regulatory interactions.

Expression quantitative trait locus analysis is an approach that has been used to investigate genes responsible for risk mechanisms attributable to intergenic loci.⁶⁸ However, expression quantitative trait locus analysis relies on the genetic variants having a quantitative effect on mRNA abundance of sufficient magnitude to be detected with a suitably stringent level of statistical significance.⁶⁹ Other mechanisms can affect disease risk without substantially altering the total amount of mRNA, such as protein localization at a functional site, as appears to be the case for EMSY. Furthermore, EMSY has at least 7 isoforms produced by alternative splicing, as well as 14 annotated phosphorylation or glycosylation sites (<https://www.uniprot.org/uniprot/Q7Z589>); it is likely that these mechanisms also contribute to functional regulation in different cell and tissue types.

Analysis of a single cell type, the keratinocyte, at different stages of terminal differentiation represents a reductionist view of the epidermis, but the skin organotypic model has proved to be a valuable model for investigation of molecular mechanisms controlling differentiation in this multilayered tissue.^{40,70} A limitation to the model is that the various dermal appendages (eg, hair follicles, sweat glands, and sebaceous glands) are absent, as are the dermal blood vessels and neurons, although these might play a role in skin inflammation.^{71,72} Immune cells of the hematopoietic lineage are also missing from this model, but there is increasing recognition of the role of keratinocytes in immune signaling,^{73,74} and the organotypic culture displays innate immunologic mechanisms.

The work presented here has identified EMSY as a therapeutic target: knockdown *in vivo* is predicted to improve skin barrier function and protect against AD. However, considerable further work is needed, including high-throughput screening to identify molecules capable of reducing the abundance of nuclear EMSY, followed by preclinical testing, before these findings can be assessed in clinical trials. The advent of biological therapy to target the immune component of AD has transformed care for patients with moderate-to-severe disease,⁷⁵ but therapies designed to improve skin barrier function are also required as an alternative or complementary approach for this complex and therapeutically challenging disorder.

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The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository (<https://www.ebi.ac.uk/pride/archive/>) with the dataset identifier PXD014088; processed data are available in the Encyclopedia of Proteome Dynamics (<https://www.peptack.com/accounts/login/epd/>); selected analysis results are available via FigShare (DOI 10.6084/m9.figshare.8088617 and 10.6084/m9.figshare.8088611). Materials are available within ethical constraints and with an MTA by request to the corresponding authors.

Key messages

- Genetic risk loci offer the opportunity for insight into the cause of complex disease, but the mechanisms require detailed molecular investigation.
- The AD-associated locus on chromosome 11q13.5 lies between 2 candidate genes: *EMSY* and *LRR32*.
- Our genetic, proteomic, and immunohistologic analyses have together demonstrated a role for *EMSY* expression in the control of skin barrier formation and function, which are of importance in patients with AD.
- Therefore *EMSY* represents a future therapeutic target in atopic disease.

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Identification of atopic dermatitis subgroups in children from two longitudinal birth cohorts.

Identification of atopic dermatitis subgroups in children from 2 longitudinal birth cohorts



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Background: Atopic dermatitis (AD) is a prevalent disease with variable natural history. Longitudinal birth cohort studies provide an opportunity to define subgroups on the basis of disease trajectories, which may represent different genetic and environmental pathomechanisms.

Objectives: We sought to investigate the existence of distinct longitudinal phenotypes of AD and test whether these findings are reproducible in 2 independent cohorts.

Methods: The presence of AD was examined in 2 birth cohort studies including 9894 children from the United Kingdom (ALSPAC) and 3652 from the Netherlands (PIAMA). AD was defined by parental report of a typical itchy and/or flexural rash. Longitudinal latent class analysis was used to investigate patterns of AD from birth to the age of 11 to 16 years. We investigated associations with known AD risk factors, including *FLG* null mutations, 23 other established AD-genetic risk variants, and atopic comorbidity.

Results: Six latent classes were identified, representing subphenotypes of AD, with remarkable consistency between the 2 cohorts. The most prevalent class was early-onset-early-resolving AD, which was associated with male sex. Two classes of persistent disease were identified (early-onset-persistent and early-onset-late-resolving); these were most strongly associated

with the AD-genetic risk score as well as personal and parental history of atopic disease. A yet unrecognized class of mid-onset-resolving AD, not associated with *FLG* mutations, but strongly associated with asthma, was identified.

Conclusions: Six classes based on temporal trajectories of rash were consistently identified in 2 population-based cohorts. The differing risk factor profiles and diverse prognoses demonstrate the potential importance of a stratified medicine approach for AD. (*J Allergy Clin Immunol* 2018;141:964-71.)

Key words: Atopic dermatitis, eczema, environmental, genetic, latent class analysis, PIAMA, ALSPAC

In clinical practice, atopic dermatitis (AD; eczema) demonstrates a characteristic itchy erythematous rash¹ but it has a heterogeneous presentation with variations in timing of onset, persistence,^{2,3} distribution, severity, association with allergic sensitization,⁴ and comorbidity with other atopic diseases.^{5,6} Although the classification of eczema cases into atopic and nonatopic forms is commonplace (in part because the underlying etiology of these may be different⁴), the heterogeneity of longitudinal disease course in AD is less well studied. Most AD

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Abbreviations used

AD: Atopic dermatitis
ALSPAC: Avon Longitudinal Study of Parents and Children
FLG: Gene encoding filaggrin
LLCA: Longitudinal latent class analysis
OR: Odds ratio
PIAMA: Prevention and Incidence of Asthma and Mite Allergy

cases are diagnosed in early childhood and although most resolve during childhood, some persist into adulthood. We hypothesized that divergent temporal disease patterns may be caused by different genetic and environmental etiological mechanisms. Understanding these differences could influence how AD is defined and treated, paving the way for a phenotype-driven, more personalized approach to the management of childhood AD.

AD is a strongly heritable condition. A total of 31 risk loci have been identified in genetic association studies, including 24 loci that were discovered in white European populations.⁷⁻¹³ The cardinal feature of an itchy erythematous rash is central to all case definitions for AD, but large genetic studies have used a broad case definition, including self-reported AD over a wide age range of pediatric and adult patients. This broad case definition has been necessary to allow collection of the large number of cases required for genome-wide analyses, but it does not allow for detailed substratification of AD and dictates that such studies are powered to detect variants common across subtypes of disease, while potentially missing variants with more specific effects on subtypes of the disease.

The aim of our study was to investigate the existence of longitudinal subphenotypes of AD and to test whether these findings are reproducible in 2 independent birth cohorts. We used longitudinal latent class analysis (LLCA), a statistical technique that can be used to model potential subgroups within a data set, to identify different longitudinal patterns of disease. We applied LLCA to cohorts from the United Kingdom and the Netherlands from birth to 16 years or 11 years, respectively. We tested each latent class for association with known genetic and nongenetic risk factors for AD and atopic comorbidities, to investigate the existence of distinct subgroups of disease having different etiological and prognostic profiles.

METHODS

Avon Longitudinal Study of Parents and Children

The Avon Longitudinal Study of Parents and Children (ALSPAC) is a longitudinal population-based birth cohort study of 14,701 children from Avon, United Kingdom. The study protocol has been described previously¹⁴ and further details are in this article's Online Repository at www.jacionline.org.

Information regarding the presence/absence of rash consistent with AD was extracted from questionnaires completed by the mothers when the children were aged between 6 months and 16.5 years (at 6, 18, 30, 42, 57, 69, 81, 103, 128, 140, 166, and 198 months). At each time point AD was defined as a positive response to 1 of the following questions: "Has your child had an itchy, dry, oozing or crusted rash on the face, forearms or shins?" (at age 6 months); "Has your child had a skin rash in the joints and creases of their body (e.g. behind the knees, elbows, under the arms) in the past 6-12 months?" (18-166 months); "Has your child had an itchy rash which was coming and going for at least 6 months in the past 12 months and confined to the creases of the knees/ankles/elbows or wrists?" (at 16 years).

Nongenetic risk factors were selected on the basis of existing evidence for association with AD¹⁵ and data availability in the 2 cohorts. Parental history of AD and asthma was parent-reported in questionnaires completed by the parents or guardians. Breast-feeding was coded as a binary variable of never versus any breast-feeding as reported by the mother when the child was 15 months old. Cat exposure was coded as a binary variable of 0 or 1+ cats in the home, as reported by the mother at 8 weeks' gestation. Children were classified as asthmatic at 7 and 13 years if a parent answered "yes" to "Has your child had asthma in the past 12 months?" Total IgE level was measured in venous blood at 7 years and total IgE level of more than 75 kU/L was defined as elevated.

DNA was obtained from blood and genotypes were determined according to methods described in this article's Online Repository at www.jacionline.org. Individuals were categorized into 2 groups: those with and those without any of the 4 gene encoding filaggrin (*FLG*) null mutations tested (ie, *FLG*^{-/-} and *FLG*^{+/-} vs *FLG*^{+/+}).¹⁶ Genotypes for the remaining 23 established (and replicated) European AD risk variants¹³ were combined into a score, with the value representing a sum of the risk alleles carried across the 23 variants.

Prevention and Incidence of Asthma and Mite Allergy

Prevention and Incidence of Asthma and Mite Allergy (PIAMA) is a Dutch multicenter birth cohort of 3963 children from allergic and nonallergic mothers. The study protocol has been described previously¹⁷ and further details are in this article's Online Repository at www.jacionline.org.

The International Study of Asthma and Allergies in Childhood-based questionnaires were used to report symptoms of AD between age 3 months and 11 years (3, 12, 24, 36, 48, 60, 72, 84, 96, 132 months). At each time point, AD was defined as a positive response to both of the following 2 questions: "Has your child had an itching rash that was variably present in the last 12 months?" (or ever at 3 and 12 months) and "Was this rash present around the eyes/ears, foreside ankles, inner side knees or inner side elbows?" (also neck at 3 and 12 months).

Parental history of asthma was taken from questionnaires that asked "Have you ever had asthma?" Any versus never breast-feeding was assessed by questions on infant feeding in the questionnaires administered at age 3 months and 1 year. Cat exposure was coded as a binary variable of 0 or 1+ cats in the home at 3 months after birth. Asthma at 7 and 11 years was defined as a parental report of a doctor's diagnosis of asthma at any time and a parental report of asthma in the last 12 months at age 7 and 11 years. Total IgE level was measured in venous blood at age 8 years and a level of more than 75 kU/L was defined as elevated.

DNA was obtained from blood and mouth swabs and genotypes were determined according to methods described in this article's Online Repository. *FLG* genotype categorization and the non-*FLG* genetic risk score were constructed, as for ALSPAC.

Statistical analysis

LLCA was used to investigate heterogeneity in patterns of AD. As the name suggests, this method is applied in longitudinal settings,^{18,19} where the aim is to identify distinct subgroups in longitudinal multivariate categorical data. This is akin to cluster analysis, but is more appropriate for binary data and allows for assignment based on probability, rather than definitive partitioning of individuals into classes. Starting with a single latent class, additional classes are added until measures that estimate model fit are optimized. Several statistical criteria (including low Bayesian information criterion, Vuong-Lo-Mendell-Rubin likelihood ratio test, and entropy) were assessed to determine the optimal number of classes; full details are given in this article's Online Repository at www.jacionline.org. Model fitting was carried out in Mplus version 7.0.²⁰

Model fit was primarily assessed using only those individuals for whom there was no missing AD symptom data. However, to optimize the use of available data and maximize the cohort size, results were compared with analyses that included individuals for whom data were available for 50% or more of the time points studied (≥ 6 of the 12 time points in the ALSPAC cohort and ≥ 5 of the 10 time points in the PIAMA cohort). Association analyses primarily focused on this larger (although incomplete) data set, but results were compared with models from the smaller but more complete data set.

Associations of risk factors and comorbidities with the latent classes were tested using a manual implementation of the bias-adjusted 3-step analysis.²¹ This method accounts for uncertainty in class assignment (see this article's Online Repository at www.jacionline.org). Associations with established risk factors (sex, family history of atopy, breast-feeding, presence of pet cat in the household, *FLG* loss-of function mutation, genetic risk score of 23 established white European AD variants) were tested using multinomial regression, whereas atopic comorbidities (asthma at ages 7 and 11 or 13 years; elevated IgE level at age 7 or 8 years) were tested using logistic regression.

RESULTS

LLCA in the ALSPAC cohort

The prevalence of AD in the ALSPAC cohort declined over time (Fig 1, A) from 27% in the first year of life to 7% at age 16.5 years. Data were available from all 12 time points for a total of 3480 individuals. The 6-class model was considered the best fit to the data (as defined by the lowest Bayesian information criterion); however, only small improvements were seen between the 4-class and the 6-class models (see Table E1 and Fig E1 in this article's Online Repository at www.jacionline.org). We present the results of the 6-class model as the primary analysis, but show the results for the simpler 4-class model in this article's Online Repository at www.jacionline.org, which for most analyses produced very similar results.

A total of 9894 individuals had data available from at least 6 of the 12 time points and the model fit parameters were broadly consistent with the smaller but more complete data set (Table E1). Comparison of models from the larger incomplete and smaller but complete data sets showed that the prevalence patterns of AD by class were very similar (see Table E3 in this article's Online Repository at www.jacionline.org) and only 3% of children (116 of 3480) changed best-fit class between the 6-class models in each analysis (see Table E2 in this article's Online Repository at www.jacionline.org).

Fig 2, A, shows the estimated prevalence of a rash characteristic of AD at each time point across the 6 classes in the analysis of 9894 individuals. Descriptions of the classes alongside the labels we gave each class are given in Table I.

The estimated prevalence of a rash characteristic of AD at each time point for the 4-class model is displayed in Fig E3 in this article's Online Repository at www.jacionline.org. The 4 classes can be described as follows: unaffected individual or transient AD (61.9%); early-onset-persistent AD (10.7%); early-onset AD resolving by age 11 years (16.5%); later-onset AD after age 3.5 years (10.9%). These 4 classes show substantial overlap with the 6-class assignment (see Table E4 in this article's Online Repository at www.jacionline.org).

LLCA in the PIAMA cohort

The prevalence of AD in the PIAMA cohort declined only slightly from 18% in the first year of life to 14% by age 11 years (Fig 1, B). Data were available from all 10 time points for 2063 individuals. A total of 3652 individuals had data available from at least 5 of these time points, and we present the results from the analysis of this larger incomplete data set. LLCA model fit was similar to ALSPAC, with lowest Bayesian information criteria achieved between the 4-class and 6-class models (see Table E5 and Fig E2 in this article's Online Repository at www.jacionline.org), the resulting class patterns following a remarkably similar pattern to ALSPAC (Fig 2, B; see Fig E4 in this

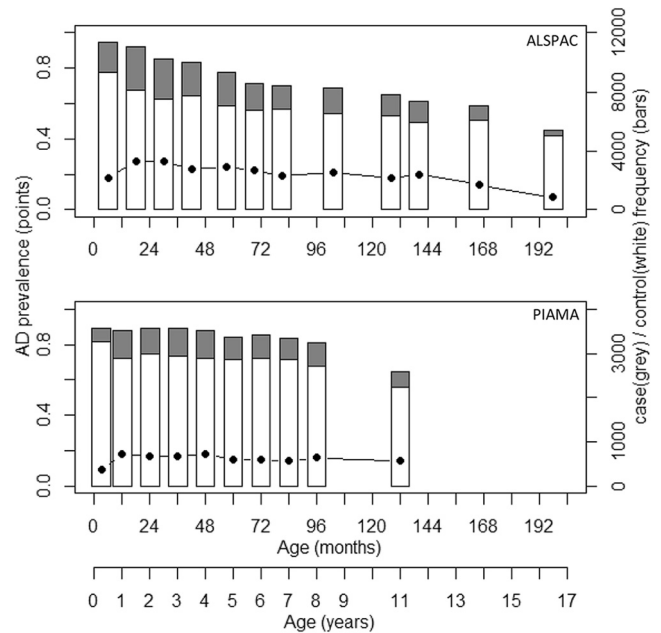


FIG 1. Prevalence and frequency of AD in UK and Dutch longitudinal cohorts. Plot of frequency (right-hand axis) of AD cases (gray bars) and controls (white bars) and AD prevalence (black points and left-hand axis) over 12 time points in ALSPAC and 10 time points in PIAMA. AD is defined by the presence of typical rash.

article's Online Repository at www.jacionline.org, with comparable class prevalences (Table I and Fig 1, B).

As for ALSPAC, we present association results for the 6-class model as the primary analysis because this showed best fit. Comparison of assignment between 4- and 6-class models is shown in Table E7 in this article's Online Repository at www.jacionline.org and association results from the 4-class model are also shown in this article's Online Repository.

Associations between latent classes with family history and selected environmental risk factors

The associations of 6 classes with potential AD risk factors are summarized in Table II. The results from the smaller but complete data set and the 4-class models are presented in Tables E9 and E10 in this article's Online Repository at www.jacionline.org. Similar conclusions could be drawn from these models, unless otherwise specified.

In ALSPAC, taking the "unaffected or transient AD" class as the baseline category, being female was a risk factor for the early-onset-persistent, mid-onset, and late-onset classes, the strongest association being with the late-onset class (odds ratio [OR], 1.90; 95% CI, 1.48-2.44; $P = 4 \times 10^{-7}$). However, male sex was a risk factor for the early-onset-early-resolving class (OR, 1.33; 95% CI, 1.10-1.61; $P = .004$). A similar pattern was observed in PIAMA, where the strongest association with female sex was observed with the late-onset group (OR, 1.87; 95% CI, 1.21-2.90; $P = .005$) and there was evidence of an association between male sex and early-onset-early-resolving class.

Maternal history of AD was associated with all classes in ALSPAC, with the strongest association in the persistent class (OR, 3.16; 95% CI, 2.60-3.83; $P = 4 \times 10^{-31}$). A similar pattern (albeit with weaker evidence for all classes) was observed in ALSPAC for



FIG 2. Longitudinal classes identified using LLCA in 2 independent birth cohorts: **A**, ALSPAC ($n = 9894$) and **B**, PIAMA ($n = 3652$).

maternal history of asthma, where again the strongest association was with the persistent class (OR, 1.54; 95% CI, 1.22-1.95; $P = 3 \times 10^{-4}$). Paternal history of asthma showed a similar association with this class (OR, 1.59), but the smaller sample size meant there was less evidence for this result ($P = .139$). Paternal asthma also showed association with the early-onset-late-resolving class (OR, 2.53; 95% CI, 1.30-4.91; $P = .006$). In PIAMA the associations with maternal and paternal history of asthma were similar, with strong associations with the persistent and early-onset-late-resolving groups for maternal history (OR, 1.94, 95% CI, 1.11-3.40, $P = .021$, and OR, 3.14, 95% CI, 1.76-5.61, $P = 1 \times 10^{-4}$, respectively) and with the persistent group for paternal history (OR, 2.69; 95% CI, 1.66-4.36; $P = 6 \times 10^{-5}$).

In ALSPAC, breast-feeding was associated with a higher risk of persistent and early-onset-late-resolving AD (OR, 1.42, 95% CI, 1.11-1.81, $P = .006$, and OR, 1.53, 95% CI, 1.12-2.08, $P = .008$, respectively). There was little evidence of association with mid- or late-onset classes. In PIAMA, there was little evidence for breast-feeding being associated with any class.

Early-life exposure to a pet cat was not associated with any of the latent classes in the primary analyses for ALSPAC or PIAMA. However, this was the only risk factor in which a difference was seen in the complete-case results in ALSPAC, where there was some evidence of a protective effect of early-life cat exposure on the early-onset-early-resolving class only (OR, 0.64; 95% CI,

0.46-0.90; $P = .010$; Table E9). The same direction of effect was observed in PIAMA but with a weaker and less precise estimate (OR, 0.72; 95% CI, 0.50-1.04; $P = .081$).

Associations between latent classes and atopic traits and comorbidities

The associations of AD classes with elevated total IgE and asthma are displayed in Table III. Raised IgE level was associated with the AD classes showing prevalent disease at the time of testing, that is, age 7 to 8 years (the persistent, early-onset-late-resolving, and mid-onset classes in ALSPAC and the persistent class in PIAMA).

In ALSPAC, all classes showed association with asthma at age 7 and 13 years. The associations were strongest for the persistent class (7 years: OR, 5.50, $P = 5 \times 10^{-41}$; 13 years: OR, 7.19; $P = 3 \times 10^{-46}$) in which 29% reported asthma at age 7 years (compared with 8% of the normal/transiently affected class), increasing to 31% at 13 years (compared with 7% of the normal/transient class). The early-onset-early-resolving class showed the smallest increased risk of asthma at age 7 and 13 years (ORs, 1.56 and 1.79, respectively). In PIAMA, the persistent and early-onset-late-resolving group showed association with asthma at age 7 years (persistent OR, 14.27; $P = 5 \times 10^{-15}$). At age 11 years, all but the mid-onset-resolving group were associated, again the strongest

TABLE I. Descriptions and prevalences of the classes in 2 independent cohorts

Class	Description of class in ALSPAC	ALSPAC prevalence	PIAMA prevalence
Unaffected individuals or transient AD	64% of this class never had reported rash, others had 1 or 2 isolated occasions of rash; ~10% reported rash consistent with AD at 6-18 mo and this declined with age	58.0%	62.9%
Early-onset-persistent AD	At age 30 mo, ~85% of this class had reported rash, increasing to >90% prevalence until 12 y; it then steadily declined to ~50% at 16.5 y	7.3%	4.9%
Early-onset-late-resolving AD	In this class the prevalence of rash rose steeply to >95% at 30 mo and then steadily declined to ~10% by 16.5 y	7.0%	3.8%
Early-onset-early-resolving AD	~60% of children in this class had reported rash at 18 and 30 mo; this declined to 10% by 6-7 y	12.9%	15.4%
Mid-onset-resolving AD	In this class there was a 10%-20% prevalence of rash until 30 mo, steeply rising to 75% prevalence at 5-6 y, and steadily declining to <10% prevalence by 16.5 y	7.0%	6.5%
Late-onset-resolving AD	In this class, ~30% reported rash at 18 mo, declining to ~10% prevalence at 5-6 y, steadily rising to ~70% prevalence by 12 y and finally declining to 10% by 16.5 y	7.9%	6.5%

association being with the persistent group (OR, 15.35; $P = 3 \times 10^{-11}$).

nucleotide polymorphism in PIAMA (OR, 0.74; 95% CI, 0.45-1.20; $P = .218$).

Associations between latent classes and genetic risk variants

In ALSPAC, all classes other than the mid-onset class showed association with *FLG* null mutations (Table IV). The strongest association was for the persistent group (OR, 4.31; 95% CI, 3.29-5.63; $P = 2 \times 10^{-26}$); the other associated classes had ORs of about half this (2.14-2.30). In PIAMA, only the early-onset-late-resolving class was associated with *FLG* null mutations (OR, 5.63; 95% CI, 2.65-11.95; $P = 7 \times 10^{-6}$); however, the approximate number of *FLG*^{-/+} or *FLG*^{-/-} individuals in the PIAMA analyses was very low (between 7 and 14 individuals per class), so power was limited to identify associations.

The combined genetic risk score encompassing all other AD variants was associated with all but the early-onset-early-resolving and the late-onset classes in the 6-class model in ALSPAC. The association was strongest with the persistent class (OR, 1.17; 95% CI, 1.12-1.22, for each additional risk allele; $P = 2 \times 10^{-13}$). A similar pattern was observed in PIAMA, with the persistent class showing the strongest association and an almost identical effect size to that seen in ALSPAC (OR, 1.17; 95% CI, 1.07-1.28; $P = 5 \times 10^{-4}$).

The associations for individual AD risk single nucleotide polymorphism are presented in Table E11 in this article's Online Repository at www.jacionline.org. These analyses are not well powered and should be interpreted with caution, but some patterns are noteworthy. Most variants had the strongest effects in the persistent class and 3 variants showed consistent associations in ALSPAC and PIAMA: These were rs17881320 in *STAT3*, rs479844 near *OVOL1*, and rs6010620 in *RTEL1*. One variant (rs1057258) showed evidence in ALSPAC for association in the opposite direction to that reported previously for AD with the late-onset and early-onset-early-resolving classes (OR, 0.73, 95% CI, 0.57-0.93, $P = .011$, and OR, 0.80, 95% CI, 0.65-0.99, $P = .039$, respectively). A consistent direction of effect (though with weak statistical evidence) was observed for the late-onset class and this single

DISCUSSION

Our results provide novel insights into the heterogeneity of AD in childhood. We report 6 latent classes, representing subphenotypes of AD with remarkable consistency between 2 independent cohorts. The most prevalent class was early-onset-early-resolving AD (13%-15%), which was associated with male sex. This class has a favorable prognosis and is only very weakly associated with asthma in later life. Two classes of persistent disease were identified (early-onset-persistent and early-onset-late-resolving); these were most strongly associated with an AD-genetic risk score as well as personal and parental history of atopic disease. Importantly, these classes display strong comorbidity with asthma. A yet unrecognized class of mid-onset-resolving AD, not associated with *FLG* mutations, but strongly associated with asthma, was described. In this class, AD prevalence increases sharply from age 2.5 years peaking at approximately 6 years. The etiological factors in this class remain unclear because the subgroup was not strongly associated with many of the known risk factors, but does show strong association with asthma comorbidity.

The clinical application of this LLCA is based on the clear demonstration of distinct classes of AD phenotype with different disease trajectories. The substantial diversity of disease that is defined as "AD" (or "eczema") has long been recognized, and clearer subdivisions are an essential prerequisite for the development of stratified medicine approaches that will be needed for the optimal application of novel biological therapies in the more severe subgroups of AD. Therefore, further studies are needed to define the most appropriate combinations of biomarkers and risk factors to detect these subgroups prospectively and at an early age.

There was some evidence of differential strength and presence of associations with risk factors and comorbidities between the classes. The early-onset-persistent class showed the strongest associations (compared with other classes) with most well-established risk factors and markers of severe atopic phenotype, including *FLG* null mutations and a genetic risk score of other

TABLE II. Association results between risk factors and AD classes identified by LLCA

Trait	Exposed/ Total	Wald <i>P</i>	Early-onset persistent	Early-onset late- resolving	Early-onset early- resolving	Mid-onset resolving	Late-onset resolving
ALSPAC			7.3%	7.0%	12.9%	7.0%	7.9%
Female	4805/9875	6×10^{-17}	1.56 (1.29-1.89) $P = 6 \times 10^{-6}$	0.97 (0.77-1.22) $P = .811$	0.75 (0.62-0.91) $P = .004$	1.79 (1.40-2.29) $P = 4 \times 10^{-6}$	1.90 (1.48-2.44) $P = 4 \times 10^{-7}$
Maternal eczema	3154/9722	3×10^{-43}	3.16 (2.60-3.83) $P = 4 \times 10^{-31}$	1.68 (1.32-2.14) $P = 2 \times 10^{-5}$	2.00 (1.65-2.44) $P = 4 \times 10^{-12}$	1.66 (1.29-2.13) $P = 8 \times 10^{-5}$	1.75 (1.36-2.25) $P = 1 \times 10^{-5}$
Maternal asthma	1554/9721	2×10^{-4}	1.54 (1.22-1.95) $P = 3 \times 10^{-4}$	1.43 (1.08-1.91) $P = .014$	1.23 (0.95-1.58) $P = .112$	1.10 (0.79-1.53) $P = .566$	1.02 (0.73-1.44) $P = .891$
Paternal asthma	245/1568	.030	1.59 (0.86-2.93) $P = .139$	2.53 (1.30-4.91) $P = .006$	1.58 (0.86-2.89) $P = .139$	0.94 (0.38-2.33) $P = .893$	1.72 (0.83-3.57) $P = .146$
Breast-feeding	7019/9198	9×10^{-4}	1.42 (1.11-1.81) $P = .006$	1.53 (1.12-2.08) $P = .008$	1.22 (0.97-1.54) $P = .093$	1.04 (0.78-1.37) $P = .803$	1.04 (0.78-1.38) $P = .800$
Pet cat	2963/9511	.179	0.88 (0.71-1.09) $P = .226$	1.14 (0.89-1.45) $P = .291$	0.92 (0.74-1.13) $P = .427$	0.81 (0.61-1.06) $P = .125$	1.26 (0.98-1.62) $P = .073$
PIAMA			4.9%	3.8%	15.4%	6.5%	6.5%
Female	1759/3652	.025	1.06 (0.75-1.49) $P = .743$	0.63 (0.40-1.00) $P = .051$	0.89 (0.64-1.24) $P = .494$	0.94 (0.65-1.37) $P = .753$	1.87 (1.21-2.90) $P = .005$
Maternal asthma	259/3645	.001	1.94 (1.11-3.40) $P = .021$	3.14 (1.76-5.61) $P = 1 \times 10^{-4}$	1.33 (0.70-2.51) $P = .385$	0.96 (0.41-2.24) $P = .932$	1.38 (0.65-2.93) $P = .406$
Paternal asthma	272/3633	.002	2.69 (1.66-4.36) $P = 6 \times 10^{-5}$	0.91 (0.34-2.46) $P = .854$	1.19 (0.63-2.25) $P = .585$	1.72 (0.94-3.14) $P = .076$	1.17 (0.53-2.61) $P = .697$
Breast-feeding	2984/3614	.888	1.00 (0.63-1.56) $P = .983$	1.34 (0.70-2.57) $P = .377$	1.19 (0.75-1.89) $P = .461$	0.97 (0.60-1.56) $P = .886$	0.88 (0.52-1.48) $P = .634$
Pet cat	1213/3651	.151	0.73 (0.50-1.06) $P = .098$	0.80 (0.50-1.29) $P = .367$	0.72 (0.50-1.04) $P = .081$	1.0 (0.68-1.47) $P = .996$	0.67 (0.42-1.07) $P = .094$

"Wald *P*" is for the overall omnibus test. Individual *P* values and effect sizes (OR and 95% CI) comparing each class with the "unaffected/transient" class are also shown; results $P < .05$ are shown in boldface.

TABLE III. Association results between AD classes identified by LLCA and comorbidities

Trait	Cases/Total	Wald <i>P</i>	Early-onset persistent	Early-onset late-resolving	Early-onset early-resolving	Mid-onset resolving	Late-onset resolving
ALSPAC			7.3%	7.0%	15.4%	7.0%	7.9%
Asthma at age 7 y	904/7859	2×10^{-50}	5.50 (4.28-7.05) $P = 5 \times 10^{-41}$	3.08 (2.22-4.27) $P = 2 \times 10^{-11}$	1.56 (1.09-2.24) $P = .015$	2.23 (1.53-3.26) $P = 3 \times 10^{-5}$	1.89 (1.26-2.83) $P = .002$
Asthma at age 13 y	784/6752	7×10^{-58}	7.19 (5.48-9.42) $P = 3 \times 10^{-46}$	3.59 (2.51-5.12) $P = 2 \times 10^{-12}$	1.79 (1.20-2.65) $P = .004$	3.41 (2.35-4.96) $P = 1 \times 10^{-10}$	2.01 (1.30-3.12) $P = .002$
Elevated IgE level at age 7 y	2057/4790	8×10^{-16}	2.62 (1.98-3.47) $P = 1 \times 10^{-11}$	1.92 (1.38-2.68) $P = 1 \times 10^{-4}$	1.15 (0.88-1.51) $P = .310$	1.55 (1.10-2.18) $P = .013$	1.38 (0.99-1.93) $P = .059$
PIAMA			4.9%	3.8%	15.4%	6.5%	6.5%
Asthma at age 7 y	94/3349	2×10^{-15}	14.27 (7.33-27.78) $P = 5 \times 10^{-15}$	5.92 (2.31-15.16) $P = 2 \times 10^{-4}$	3.03 (1.08-8.47) $P = .035$	0.60 (0.03-13.62) $P = .750$	1.73 (0.38-7.88) $P = .478$
Asthma at age 11 y	102/2639	7×10^{-11}	15.35 (6.86-34.35) $P = 3 \times 10^{-11}$	9.12 (3.49-23.82) $P = 6 \times 10^{-6}$	4.91 (1.66-14.55) $P = .004$	2.10 (0.42-10.53) $P = .366$	5.70 (1.98-16.43) $P = .001$
Elevated IgE level at age 8 y	723/1707	1×10^{-4}	3.00 (1.85-4.86) $P = 8 \times 10^{-6}$	1.58 (0.86-2.89) $P = .140$	1.57 (0.97-2.56) $P = .067$	1.42 (0.83-2.46) $P = .203$	0.97 (0.53-1.77) $P = .914$

"Wald *P*" is for the overall omnibus test. Individual *P* values and effect sizes (OR and 95% CI) comparing each class with the "unaffected/transient" class are also shown; elevated IgE level is defined as total IgE level of >75 kU/L; results $P < .05$ are shown in boldface.

AD-associated variants, coexistent asthma, and elevated IgE and parental history of atopic disease. The associations with asthma at ages 7 and 11 and 13 years were strongest with the persistent class, but all AD classes showed evidence of some increased risk of asthma at these ages. Our data did not support the presence of a specific trajectory from AD to asthma (the so-called atopic march), which is in keeping with a previous report from the Manchester Asthma and Allergy Study (MAAS) and earlier analyses in ALSPAC.⁶ The associations observed with elevated total IgE level were most marked during active and persistent disease, in

keeping with previous reports.²² Within the class of early-onset disease that resolved before the time of IgE measurement, a smaller proportion of individuals had IgE levels above the threshold defined as "elevated," in comparison with the class of early-onset disease that was still active. Further investigation with earlier IgE measures is required to explore whether such individuals would have had raised IgE level at the time of active disease.

Although being female was more strongly associated with the early-onset persistent and late-onset classes, there was some

TABLE IV. Association results between genetic risk factors and AD classes identified by LLCA

Study and genetic risk factor	No. with risk genotype/total	Wald <i>P</i>	Early-onset persistent	Early-onset late-resolving	Early-onset early-resolving	Mid-onset resolving	Late-onset resolving
ALSPAC			7.3%	7.0%	12.9%	7.0%	7.9%
FLG null mutation	813 of 7570	4×10^{-28}	131 of 570 (23%)* 4.31 (3.29-5.63) $P = 2 \times 10^{-26}$	78 of 514 (15%)* 2.23 (1.53-3.26) $P = 3 \times 10^{-5}$	111 of 832 (13%)* 2.14 (1.54-2.98) $P = 7 \times 10^{-6}$	54 of 467 (12%)* 1.48 (0.92-2.39) $P = .109$	70 of 499 (14%)* 2.30 (1.57-3.38) $P = 2 \times 10^{-5}$
Genetic risk score (all other variants)	Total N = 6497	8×10^{-17}	1.17 (1.12-1.22) $P = 2 \times 10^{-13}$	1.08 (1.04-1.13) $P = 4 \times 10^{-4}$	1.02 (0.99-1.06) $P = .222$	1.06 (1.00-1.12) $P = .042$	1.01 (0.96-1.06) $P = .758$
PIAMA			4.9%	3.8%	15.4%	6.5%	6.5%
FLG null mutation	117 of 1516	6×10^{-4}	7 of 74 (10%)* 1.34 (0.49-3.67) $P = .563$	14 of 60 (23%)* 5.63 (2.65-11.95) $P = 7 \times 10^{-6}$	11 of 159 (7%)* 0.87 (0.25-3.03) $P = .821$	7 of 96 (7%)* 0.95 (0.26-3.45) $P = .942$	10 of 95 (11%)* 1.87 (0.76-4.62) $P = .174$
Genetic risk score (all other variants)	Total N = 1964	6×10^{-5}	1.17 (1.07-1.28) $P = 5 \times 10^{-4}$	1.0 (0.91-1.11) $P = .968$	1.16 (1.08-1.25) $P = 1 \times 10^{-4}$	1.11 (1.03-1.20) $P = .004$	1.08 (0.98-1.18) $P = .111$

"Wald *P*" is for the overall omnibus test. Individual *P* values and effect sizes comparing each class with the "unaffected/transient" class are also shown; results $P < .05$ are shown in boldface; genetic risk score is defined by the total number of risk alleles across the 23 AD-associated loci (other than *FLG*) identified by genome-wide association study meta-analysis to date; OR represents the change in odds per risk allele for the genetic risk score or between carriers compared with noncarriers for the *FLG* mutations.

*To demonstrate the approximate numbers of individuals with *FLG* null mutations in each class, individuals were assigned to the most likely class. Given that the actual association analysis accounted for uncertainty in assignment of classes, these values are approximations for purposes of highlighting where power might be low. The approximate number with *FLG* null mutations/approximate total with *FLG* genotype data (%), within each class are shown. The "unaffected/transient" groups had 8% (~369 of 4712) and 7% (~68 of 1032) with *FLG* mutations in ALSPAC and PIAMA, respectively.

evidence that being male was differentially associated with early-onset-resolving classes. It is tempting to speculate that the late-onset class might represent AD induced by behavioral changes in the adolescent child (including increased bathing/showering and the use of fragranced products), which might differ between males and females, but this hypothesis remains to be tested. The male preponderance in AD cases ascertained in infancy has previously been reported²³⁻²⁶ but the mechanisms accounting for this sex difference are unknown.

There is conflicting epidemiological evidence indicating that breast-feeding may be either a risk factor or a protective factor in the etiology of AD²⁷ and our analyses have not been able to add clarity to this important question. In the ALSPAC cohort, breast-feeding was associated with the 2 classes of most long-lasting disease (early-onset-persistent and early-onset-late-resolving AD) but in the PIAMA cohort there was little evidence of breast-feeding being associated with any of the latent classes. This apparent difference could be explained in several different ways: It may be stochastic (given that all 95% CIs overlap between the 2 cohorts); it may be a result of the substantially higher prevalence of breast-feeding in the Dutch population compared with the UK population; or it may result from reverse causation in the ALSPAC cohort, if, for example, mothers with a strong history of atopic disease are more likely to breast-feed their infants.

We found little evidence for early-life exposure to a pet cat being associated with any of the classes in the main analysis. However, in the complete-case data set in ALSPAC, there was some evidence that cat exposure may have a protective effect on early-onset-early-resolving AD, which is somewhat at odds with previous reports of cat exposure increasing the risk of AD.^{28,29} This may indicate a specific beneficial effect of early cat exposure on this more transient phenotype and warrants further investigation.

There was evidence that *FLG* null mutations were associated with all classes; however, as reported previously,^{30,31} the association was strongest in the group with early-onset-persistent

disease. The genetic risk score of the other established AD variants showed a similar pattern, whereby the association was strongest for the early-onset-persistent class, with a striking increase in the burden of risk of approximately 17% per additional risk allele. Of note, 3 variants showed consistent patterns of effects across both cohorts, with stronger associations in the early-onset persistent group, and weaker associations with the other classes. The functional mechanisms of these loci have not been fully defined, but rs17881320 is within *STAT3* (encoding a signal transducer and activator of transcription, an acute-phase response protein); rs479844 is near to *OVOLI* (which encodes zinc-finger containing transcription factor); and rs6010620 is within *RTEL1* (regulator of telomere elongation helicase 1). This heterogeneity of effect of genetic variants on different disease profiles emphasizes the need for patient stratification in future genetic studies. Stratification may be used to increase the power to detect variants associated with specific classes; stratification could also allow the identification of phenotype-specific mechanistic pathways as future therapeutic targets.

The similarity and high frequency of AD symptom ascertainment in ALSPAC and PIAMA are strengths of our study. The phenotype definitions used within the cohorts comprised prospective questions to capture diagnostic features of eczema including the changing distribution of skin involvement from infancy to later childhood. One key difference is that PIAMA had a shorter follow-up (11 years vs 16 years), which could have limited the ability to detect classes with differences at later ages. Despite this, the class patterns are remarkably similar between ALSPAC and PIAMA (Fig 2). However, neither cohort allowed for investigation of variable AD patterns in adulthood, more subtle AD patterns (such as the transient cases that were indistinguishable from the unaffected individuals in our study), nor in people of ancestries other than white European. We also studied only a limited number of environmental factors and so this work could be extended to investigate the association between latent classes and other potential risk factors.

Although individuals are not assigned to classes with complete certainty (see Table E8 in this article's Online Repository at www.jacionline.org), the LLCA 3-step method models this uncertainty and allows for the inclusion of individuals with incomplete data to maximize sample size and minimize any loss to follow-up bias. We note that our analysis does not formally test for causal direction and some risk factors studied do not entirely precede the onset of disease. Therefore, although the observational associations are interesting, further work should be conducted to investigate causality. A further challenge is that by stratifying AD into smaller subphenotypes of disease, we inevitably lower the power of association testing. Few studies have such detailed longitudinal data and so to increase sample sizes in future studies, it will be necessary to extrapolate these data-driven phenotypes into settings where less detailed data are available, such as large data registries.³²

In conclusion, we have identified longitudinal subgroups of AD that have both shared and distinctly different risk factor profiles. Future studies of the etiology and treatment of this complex trait should take these subgroups of disease into account and, in turn, this may offer valuable stratified medicine approaches to refining prognostic predictions and therapeutic strategies.

ALSPAC: We are extremely grateful to all the families who took part in this study, the midwives for their help in recruiting them, and the whole ALSPAC team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists, and nurses. Genome-wide genetic data were generated by Sample Logistics and Genotyping Facilities at Wellcome Sanger Institute and LabCorp (Laboratory Corporation of America) using support from 23andMe. PIAMA: We thank all the children and their parents for their cooperation. We also thank all the field workers and laboratory personnel involved for their efforts, and Marjan Tewis for data management. This publication is the work of the authors and L.P. will serve as guarantor for the contents of this article.

Clinical implications: AD ranges from a transient condition to lifelong morbidity. This study has identified distinct subphenotypes of AD in children, which could indicate the importance of a stratified approach to the management of this complex disease.

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Genome-wide comparative analysis of atopic dermatitis and psoriasis gives insight into opposing genetic mechanisms.

Genome-wide Comparative Analysis of Atopic Dermatitis and Psoriasis Gives Insight into Opposing Genetic Mechanisms

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Atopic dermatitis and psoriasis are the two most common immune-mediated inflammatory disorders affecting the skin. Genome-wide studies demonstrate a high degree of genetic overlap, but these diseases have mutually exclusive clinical phenotypes and opposing immune mechanisms. Despite their prevalence, atopic dermatitis and psoriasis very rarely co-occur within one individual. By utilizing genome-wide association study and ImmunoChip data from >19,000 individuals and methodologies developed from meta-analysis, we have identified opposing risk alleles at shared loci as well as independent disease-specific loci within the epidermal differentiation complex (chromosome 1q21.3), the Th2 locus control region (chromosome 5q31.1), and the major histocompatibility complex (chromosome 6p21–22). We further identified previously unreported pleiotropic alleles with opposing effects on atopic dermatitis and psoriasis risk in *PRKRA* and *ANXA6/TNIP1*. In contrast, there was no evidence for shared loci with effects operating in the same direction on both diseases. Our results show that atopic dermatitis and psoriasis have distinct genetic mechanisms with opposing effects in shared pathways influencing epidermal differentiation and immune response. The statistical analysis methods developed in the conduct of this study have produced additional insight from previously published data sets. The approach is likely to be applicable to the investigation of the genetic basis of other complex traits with overlapping and distinct clinical features.

Introduction

Atopic dermatitis (AD, synonymous with eczema [MIM 603165]) and psoriasis (psoriasis vulgaris [MIM 177900]) are the two most common chronic inflammatory skin con-

ditions. They are associated with a significantly reduced quality of life and multiple comorbidities.^{1,2} Both diseases result from the interaction of genetic and environmental factors and are characterized by epidermal defects as well as local and systemic immunological abnormalities.

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Despite a lifetime prevalence of ~2% for psoriasis and 10%–20% for AD,^{3,4} these diseases rarely co-occur within an individual⁵—an observation attributed to opposing immune response patterns.⁶ However, it has been reported that both Th1-cell-dominated autoimmune and Th2-cell-dominated allergic diseases aggregate within families⁷ and that parental psoriasis might increase the risk of AD in offspring.⁸ Furthermore, genome-wide linkage and association studies have shown genetic risk loci in each disease that map to similar regions of the genome. The epidermal differentiation complex (EDC) on chromosome 1q21.3 includes AD and psoriasis risk loci in close proximity.^{9–12} Null mutations in the gene encoding filaggrin (*FLG* [MIM 135940]) represent the strongest known risk factor for AD^{13,14} and account for at least a proportion of AD risk within the EDC, but *FLG*-null mutations are not associated with psoriasis.^{15,16} A deletion of the late cornified envelope genes *LCE3B-LCE3C* (MIM 612614, 612615) represents a genetic substrate for psoriasis within the EDC,^{17,18} but this deletion is not associated with AD.¹⁹ The cytokine cluster encoded at 5q23.1–5q31.1 includes variants showing association with both diseases,^{10,20,21} and an intergenic region of chromosome 20q13.2 has also shown association with both AD and psoriasis.^{22,23} Finally, a recent genome-wide association study (GWAS) on AD identified a strong association within the margins of the major histocompatibility complex (MHC)²⁰ on chromosome 6p21.3, less than 2.4 kb from a variant associated with HLA-Cw6 (MIM 142840),²⁴ the strongest known psoriasis-risk locus.

In order to gain insight into overlapping and specific genetic mechanisms, we systematically compared and contrasted AD and psoriasis via analytical techniques developed from meta-analysis.

Subjects and Methods

Study Subjects

Genome-wide genotype data were obtained on samples from six case-control cohorts (three each of AD and psoriasis), totaling 2,262 AD and 4,489 psoriasis case subjects and 12,333 control subjects (Table S1 available online).

The German AD case subjects were recruited from tertiary dermatology clinics at Munich, as part of the GENEVA study, University of Kiel, University of Bonn, and the University Children's Hospital of Charité Universitätsmedizin Berlin. AD was diagnosed by experienced dermatologists and/or pediatricians according to the UK Diagnostic Criteria.²⁵ German control subjects were obtained from the PopGen biorepository,²⁶ the population-based KORA study in southern Germany,²⁷ and the German part of ISAAC II to assess the prevalence of asthma and allergies in schoolchildren.²⁸ The Irish AD case collection was recruited from the secondary and tertiary pediatric dermatology clinic at Our Lady's Children's Hospital, Crumlin, Dublin. Irish control individuals were obtained from healthy adult blood donors as part of the Trinity Biobank, Dublin.²⁹

The German psoriasis case subjects were recruited from the tertiary dermatology clinic at the University of Kiel and German controls were again obtained from the PopGen biorepository and the

KORA study (independent from those used as controls for AD). The British psoriasis case-control study is part of the Wellcome Trust Case Control Consortium 2²⁴ and the US psoriasis study has been described elsewhere.²¹

ImmunoChip data on 2,425 AD case subjects, 3,580 psoriasis case subjects, and 9,061 control subjects were obtained from previous studies,^{11,12} including data on a subset of case and control individuals also analyzed by GWAS. Results of analysis of the four most prevalent *FLG* (RefSeq accession number NM_002016.1) loss-of-function mutations were obtained for a total of 2,865 case subjects and 5,540 control subjects as data generated for previous studies;^{11,20} the *FLG* mutations in these analyses are as follows: p.Arg501* (c.1501C>T), p.Ser761Cysfs*36 (c.2282_2285del), p.Arg2447* (c.7339C>T), and p.Ser3247* (c.9740C>A) (R501X, 2282del4, R2447X, and S3247X, respectively).

The institutional review board in each contributing center approved these studies. All participants (or their parents or guardians) gave written informed consent.

Study Design

The study design is summarized in Figure 1.

Quality Control

Quality control and standard GWAS analysis of genotyped single-nucleotide variants (SNVs) was carried out with PLINK³⁰ and R. Samples with extensive missing data (rate >5%), excess of heterozygosity or homozygosity, and discrepant gender determined on the basis of average X-chromosomal heterozygosity compared to the gender recorded in the database were excluded. We then examined identity-by-state (IBS) sharing and estimated identity-by-descent (IBD) on a pruned SNV set between all pairs of individuals and deleted resulting duplicates or closely related samples with PI_HAT > 0.1875 (halfway between expected IBD for third- and second-degree relatives). Multidimensional scaling (MDS) of the pairwise IBS matrix was carried out to identify and delete outliers of unusual ancestry and to calculate genome-wide principal-component scores for each individual. We excluded 894 samples because of SNVs showing a missing rate of >5%, deviation of Hardy-Weinberg equilibrium $p_{HWE} < 10^{-8}$, or minor allele frequency (MAF) < 5% (summarized in Table S2). After quality control, the resulting SNVs and samples were analyzed for association via logistic regression with age, sex, and principal-component scores as covariates. Results from each panel were investigated to determine whether established GWAS loci were identified for the respective trait of interest, and genomic control inflation factors were calculated.

Imputation of SNVs and Classical HLA Alleles

Any SNVs showing significant association were checked (e.g., by visual inspection of the intensity cluster plots and investigation of consistency of LD with surrounding markers) and those SNVs deemed unreliable were removed. The final data sets of high-quality SNVs were prephased with SHAPEIT³¹ and subsequently used to perform imputation with IMPUTE2,³² the 1000 Genomes reference panel (integrated variant set, release March 2012).³³ In the Irish AD collection (Table S1), case and control subjects were genotyped on different platforms, and therefore only the 131,692 SNVs in common between the platforms were used to inform imputation.

Postimputation SNVs with low imputation quality (info score < 0.4), call rate < 95%, deviation from $p_{HWE} < 10^{-8}$, or MAF < 5%

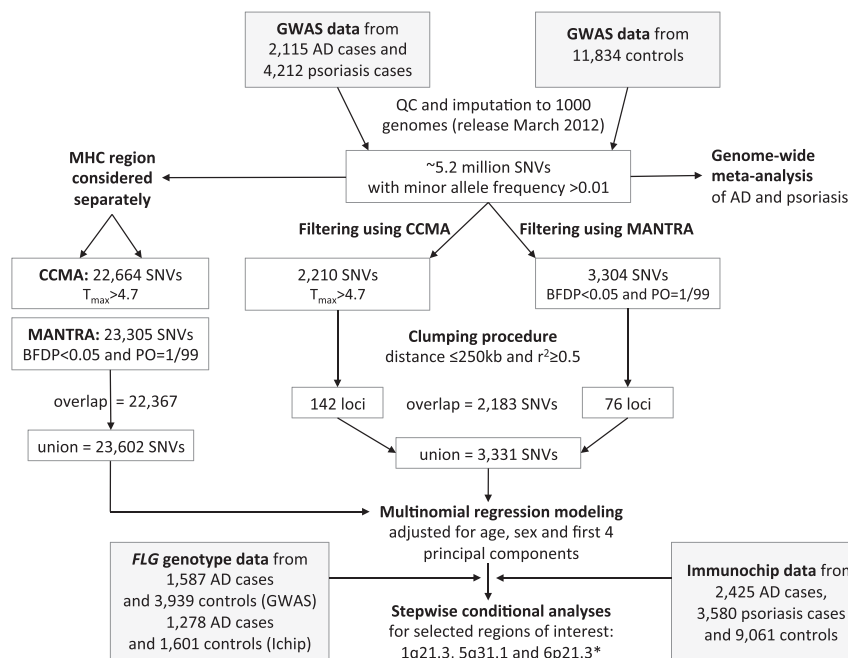


Figure 1. Study Design

Abbreviations are as follows: CCMA, case control meta-analysis; MANTRA, meta-analysis of trans-ethnic association studies; BFD, Bayesian false discovery; PO, prior odds; *conditional analysis for the MHC was also carried out with imputed classical HLA-allele (detailed in the [Subjects and Methods](#)).

were excluded. A final data set of approximately 5.2 million SNVs in 2,079 AD case subjects, 3,867 control subjects, 4,212 psoriasis case subjects, and 8,032 control subjects were eligible for subsequent analysis ([Table S3](#)).

Classical alleles for *HLA-A*, *HLA-B*, and *HLA-C* were imputed for each case-control cohort separately by HLA*IMP^{34,35} and best guess genotypes with probability >0.9. Additional classical *HLA-DQA1*, *HLA-DQB1*, and *HLA-DRB1* alleles were imputed in each case-control cohort with the exception of the Irish samples, in which there were insufficient informative SNPs. Alleles with a frequency >1% were put forward for analysis. For each individual, alleles were coded as having no, one, or two copies of the respective allele via allele probability >0.9. We obtained high-quality data at the four-digit level with call rates of 92%–100% and accuracy of 92%–98%.

Statistical Analysis

Meta-GWAS was performed on each disease, via standard methodologies. To analyze these findings further, we developed two different meta-analysis-based approaches to filter SNVs and model the contrasting effects in each disease. The first was a compare and contrast meta-analysis (CCMA) approach inspired by a subset-based method.³⁶ The second used transethnic meta-analysis implemented in the MANTRA software,³⁷ combining all six studies by using prior clustering to reflect the ethnic difference and the disease type. The MHC region was reserved for separate analysis because of its unique and complex variability and patterns of strong linkage disequilibrium (LD).

The CCMA approach is based on an adaptation of an idea of Bhattacharjee et al.,³⁶ who modeled association with heterogeneous traits. With METAL,³⁸ we calculated z-scores signed positive or negative with respect to the same reference allele for two meta-analyses, T_1 , combining AD studies only, and T_2 , combining psoriasis studies only. We then calculated the overall test statistic T_{max} with the formula $T_{max} = \max(|T_1|, |T_2|, |T_{12shared}|, |T_{12opposing}|)$, where $T_{12shared} = (T_1 + T_2)/\sqrt{2}$ and $T_{12opposing} = (T_1 - T_2)/\sqrt{2}$. We categorized the effect of each SNV as corresponding to an effect

on AD only, to an effect on psoriasis only, to a shared effect (in the same direction on AD and psoriasis), or to opposing effects, according to which of the four test statistics ($|T_1|$, $|T_2|$, $|T_{12shared}|$, $|T_{12opposing}|$) was the largest. In order to derive a p value for T_{max} , we worked out an empirical null distribution by simulating 10,000,000 realizations of two normally distributed random variables, Z_1 and Z_2 . Then we calculated $Z_{12shared} = (Z_1 + Z_2)/\sqrt{2}$, $Z_{12opposing} = (Z_1 - Z_2)/\sqrt{2}$, and $Z_{max} = \max(|Z_1|, |Z_2|, |Z_{12shared}|, |Z_{12opposing}|)$. The empirical p values can be derived as

$P_{emp} = (\#(Z_{max} > T_{max}) + 1)/(\# \text{ simulations} + 1)$.

In a separate simulation of 1,000,000,000 replicates, we derived a calibration curve for the p values and found it suitable up to a p value of 10^{-9} . Hence with the calibration curve we can derive Z_{max} thresholds corresponding to standard genome-wide “suggestive” (10^{-5}) and genome-wide “significant” (10^{-8}) thresholds, corresponding to T_{max} values of approximately 4.7 and 6.0, respectively ([Figure S1](#)).

In the second approach we used the MANTRA software³⁷ developed for transethnic meta-analysis. MANTRA uses a Bayesian partition model for grouping studies according to their ethnicity. We adopted this idea and worked out a prior distribution to cluster studies according to both our phenotypes of interest and the genetic distance between the studies derived from our MDS analysis based on the pairwise IBS matrix: $D_{Total} = D_{Disease} + D_{Ethnicity}$, where $D_{Ethnicity}$ is a diagonal matrix of Euclidean distances between study centers. To distinguish the two diseases (psoriasis and AD), we set the corresponding cells of the $D_{Disease}$ matrix to $D_{ij} = 2 \times \max(D_{Ethnicity})$ and to account for the different subphenotype in AD (AD in general versus childhood AD), we set the corresponding cells of the $D_{Disease}$ matrix to $D_{ij} = \max(D_{Ethnicity})$, resulting in the prior components shown in [Table S4](#).

We calibrated the resulting $\log_{10}BF_{MANTRA} = \log_{10}(\text{Bayes Factors})$ from the MANTRA software in order to find a threshold for filtering SNVs, which were compared with the CCMA top SNVs and subsequently carried forward to multinomial regression modeling. To perform this calibration, we calculated the Bayesian False Discovery Probability proposed by Wakefield³⁹ with diverse prior odds (PO) in favor of H_0 :

$$BFDp = \frac{BF_{MANTRA} \times PO}{1 + BF_{MANTRA} \times PO}$$

Sensitivity analysis was performed with only the $D_{Ethnicity}$ as prior matrix and we observe high correlation ($r^2 > 0.99$) of the top-ranked SNVs (BFDp < 0.05; PO = 99) with our analysis (data not shown).

Finally, we carried forward a filtered set of SNVs from CCMA and MANTRA for modeling via a multinomial regression model, adjusted for sex and the first four genome-wide principal-component scores. The multinomial model involves three outcome categories: the “baseline” category into which all controls are categorized, a “psoriasis” case category, and an “AD” case category (modeled through regression coefficients β_{PSO} and β_{AD} , respectively). This analysis makes use of individual-level genotypes and is thus more computationally intensive (although arguably more powerful and more statistically satisfactory) than CCMA and MANTRA. We calculated p values for tests that were designed to be sensitive to the following situations: an overall SNV effect (on either or both diseases, in either direction), an individual SNV effect on one disease (but not on the other), a shared SNV effect (operating in the same direction for both diseases), and a contrasting SNV effect (operating in opposing directions between both diseases), by performing Wald tests of the following linear hypotheses:

$$\text{Overall effect: } H_0: \begin{matrix} \beta_{PSO} + \beta_{AD} = 0 \\ \beta_{PSO} - \beta_{AD} = 0 \end{matrix}, H_1: \begin{matrix} \beta_{PSO} + \beta_{AD} \neq 0 \\ \beta_{PSO} - \beta_{AD} \neq 0 \end{matrix}$$

$$\text{Psoriasis effect: } H_0: \beta_{PSO} = 0, H_1: \beta_{PSO} \neq 0$$

$$\text{AD effect: } H_0: \beta_{AD} = 0, H_1: \beta_{AD} \neq 0$$

$$\text{Shared effect: } H_0: \beta_{PSO} + \beta_{AD} = 0, H_1: \beta_{PSO} + \beta_{AD} \neq 0$$

$$\text{Opposing effect: } H_0: \beta_{PSO} - \beta_{AD} = 0, H_1: \beta_{PSO} - \beta_{AD} \neq 0$$

The overall significance of the SNV was assessed through the 2 degree of freedom (df) test of overall effect, which compares the null hypothesis that the SNV has no effect on either psoriasis or AD with the alternative hypothesis that it has an effect on one or both diseases. The other four 1 df tests were used to categorize the effect (in analogy to CCMA) in four categories—AD only, psoriasis only, shared effect, and opposing effects—by categorizing according to the minimum of the p values: $p_{MNM} = \min(p_{AD}, p_{PSO}, p_{SHARED}, p_{OPPOSING})$. The rationale for the use of the minimum of these 1 df tests for categorization is as follows: if a SNV is associated with one disease but not the other, the test of a nonzero regression coefficient for that disease (even while unnecessarily also allowing for a nonzero coefficient for the other disease, as is done in the psoriasis effect and AD effect tests), should be more powerful than a test that erroneously groups together the coefficients of the associated and the nonassociated disease (as is done in the shared and opposing effect tests). This is on account of the fact that grouping together these coefficients will incur a penalty in terms of increasing the variance, while not incurring any greater expected magnitude of effect since the expected value of the regression coefficient for the nonassociated disease is zero. If, on the other hand, the SNV has effects that operate in the same direction on both diseases, then a test based on adding together these effects (as is done in the shared effect test) should be more powerful than considering each effect on its own, or subtracting one effect from the other (as is done in the opposing effect test), because adding together the coefficients induces the greatest magnitude of effect. Finally, if the SNV has effects that operate in opposite directions in the two diseases, then a test based on subtracting one effect from the other (as is done in the opposing effect test) should be most powerful because it induces the greatest magnitude of effect.

All analyses if not explicitly stated were carried out with R. For the purposes of this analysis, we distinguished between a shared genetic “region” and a shared genetic “locus.” We arbitrarily designated a shared region as a block of genomic DNA spanning 2 Mb with association signals for both traits. We defined a genetic locus as the lead SNV and all SNVs with $r^2 > 0.5$.

Predicted Protein Network Analysis and Gene Ontology Analysis

Functional protein association networks were investigated in silico and gene ontology analyses were performed with STRING_{9.1}.

Results

Filtering Variants to Define Risk Effects

Quality control and imputation generated 5.2 million SNVs with a minor allele frequency >0.01 for further analysis (Figure 1). GWASs within each cohort resulted in genomic inflation factors λ between 1.03 and 1.08. Meta-GWAS performed on each disease confirmed previously reported risk loci in AD and psoriasis and illustrated areas of colocalization on chromosomes 1, 5, and 6 (Figure 2A).

Excluding the MHC, 2,210 SNVs were identified with shared (by which we mean alleles having effects operating in the same direction in both diseases), opposing, and disease-specific SNVs with CCMA test statistic $T_{max} > 4.7$. This threshold was defined to correspond to a suggestive significance of $p < 10^{-5}$ in order to reduce the probability of false negatives. The 2,210 SNVs were condensed to 142 distinct loci after an LD-based clumping procedure³⁰ with the following parameters: distance ≤ 250 kb and $r^2 \geq 0.5$.

Analysis with MANTRA revealed 3,304 SNVs with Bayesian false discovery probability (BFDP) < 0.05 with prior odds (PO) 1/99 resulting in 76 distinct loci after clumping. The overlap of CCMA and MANTRA gave 2,183 SNVs and the union of both methods resulted in 3,331 SNVs that were carried forward for multinomial regression modeling (MNM), which was adjusted for sex and the first four genome-wide principal-component scores. The results are displayed in Figure 2B, in which disease-specific, shared, and opposing loci are coded by color. SNVs showing genome-wide significance in at least one of the three methods of analysis ($T_{max} > 6$, BFDP < 0.05 with PO = 1/999, or $p_{MNM} < 10^{-8}$) map to 144 distinct loci (Table S5). Comparison of effect classification (AD, psoriasis, shared, opposing) in CCMA and MNM (Figure S2) showed an agreement of 94.8% when excluding the MHC region (Figure S3). For further investigation, we considered only loci containing more than one SNV and an effect classified in the same direction by CCMA and MNM.

Validation of Previously Reported AD- and Psoriasis-Risk Loci

15 European and 9 Asian loci have previously been reported in GWASs on AD, and 44 European and 9 Asian loci have been reported in association with psoriasis (Table S6). In our disease-specific meta-analysis individuals of

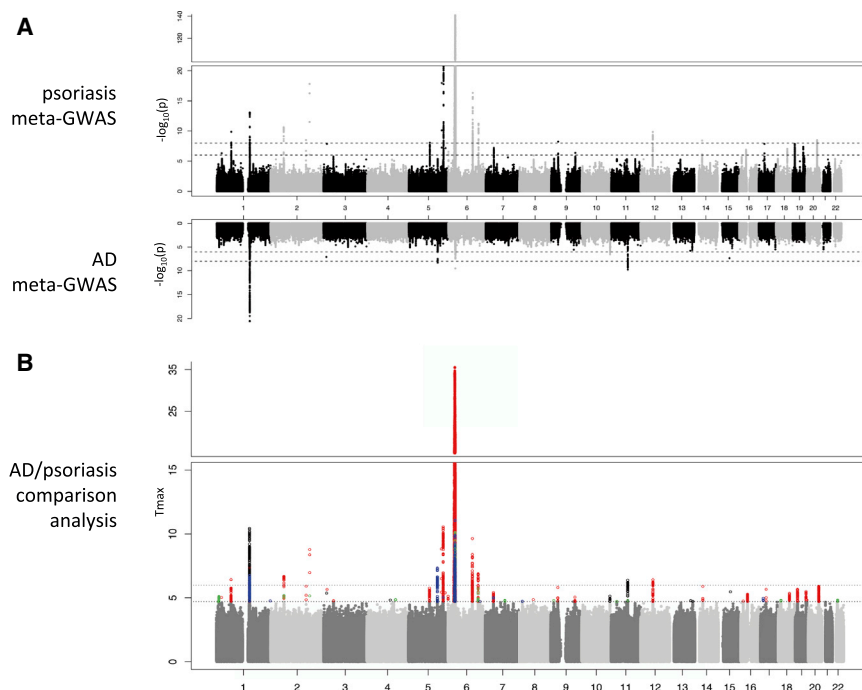


Figure 2. Genome-wide Comparison of AD and Psoriasis

(A) Mirrored Manhattan plots showing results of AD meta-GWAS (top) and psoriasis meta-GWAS (bottom).

(B) Comparative analysis of AD and psoriasis in which SNVs are color coded to show AD-specific effect (black), psoriasis-specific effect (red), shared effects defined as alleles operating in the same direction (green), and opposing effects (blue). The genome-wide significance level is marked at $p = 0.5 \times 10^{-8}$ ($T_{max} = 6.0$).

white European descent, 14 of the European AD loci as well as 43 of the psoriasis loci are replicated. Furthermore, 4 AD and 4 psoriasis loci so far reported only in Asians showed evidence for association in European populations ($p < 10^{-3}$): *CCDC80* (MIM 608298)/*CD200R1L* at 3q13.2, *CARD11* (MIM 607210) at 7p22.2, *ZNF365* (MIM 607818) at 10q21.2, and *BCAS1* (MIM 602968) at 20q13.2 in AD; *CSMD1* (MIM 608397) at 8p23.2, *SERPINB8* (MIM 601697) at 18q22.1, *MAMSTR* (MIM 610349)/*RASIP1* (MIM 609623) at 19q13.33, and *ZNF816A* at 19q13.41 in psoriasis (Table S6).

New Opposing-Effect Loci Identified by Genome-wide Comparative Analysis

Excluding the MHC, 25 loci showed a genome-wide significant association with either skin disorder, defined by all three methods of analysis (CCMA $T_{max} > 6$ and MANTRA BFD $p < 0.05$ with $PO = 1/999$, and $p_{MNM} < 10^{-8}$) including six loci that were coassociated with both AD and psoriasis. Each coassociated locus displayed opposing effects and two of these loci (2q31.2, 5q33.1) have not previously been reported as showing coassociation with AD and psoriasis (Table 1).

2q31.2 demonstrates an opposing effect at rs62176107 (MNM $p = 1.08 \times 10^{-34}$; Table S5); this variant is within exon 6 of *PRKRA* (MIM 603424) and also within microRNA 548n (MIR548N) and a noncoding transcript, AC009948.5. *PRKRA* encodes protein kinase interferon-inducible double-stranded RNA-dependent activator (PACT), a cellular dsRNA-binding protein originally identified as a binding partner and activator of PKR in response to extracellular stress.⁴⁶ More recently, it has been shown to be an essential factor in the PKR-independent initiation

of RIG-I-induced antiviral response.⁴⁷ Of note, individuals with AD are known to be susceptible to viral skin infections, but cutaneous infections rarely occur in psoriasis.⁴⁸ MicroRNAs play a role in posttranscriptional regulation of gene expression by affecting the stability and translation of mRNAs, but the specific role of miRNA548n has not been defined. The most significantly associated (“lead”) SNV at 2q31.2 (rs62176107, G>A, having the smallest p value from MNM) is a synonymous SNV with predicted effects on 12 transcripts, including *PRKRA* splice variants’ UTR and intronic regions and a variant predicted to undergo nonsense-mediated decay (Ensembl release 75). Gene expression profiling data show downregulation of both *PRKRA* mRNA and miRNA548n in psoriatic lesions compared to nonlesional skin, but no significant differences in AD (Table S7).

The most highly significant variant at 5q33.1 (rs17728338) shows opposing effects on AD and psoriasis (MNM $p = 3.96 \times 10^{-38}$; Table S5) and lies 2 kb upstream of *ANXA6* (MIM 114070) and 8 kb downstream of *TNIP1* (MIM 607714). LD analysis in 1000 Genomes (release August 2009) via LocusZoom⁴⁹ showed that rs17728338 is located within a 25-kb block containing both *TNIP1* and *ANXA6*. The locus has previously been associated with psoriasis in European and Chinese populations but has not been implicated in AD. *TNIP1* is involved in TNF signaling and regulation of the transcription factor NF- κ B;^{21,50} it shows increased expression both in AD and psoriatic lesions compared to control skin (Table S7). In contrast, *ANXA6*, which encodes a calcium-dependent membrane and phospholipid binding protein, shows significant upregulation of expression in atopic skin compared to control skin (fold change 1.3, FDR $p = 0.016$) and lesional to nonlesional AD skin (fold change 2.4, $p = 0.027$), whereas expression is decreased in psoriatic versus healthy skin (fold change 0.7, $p = 6.38 \times 10^{-13}$) (Table S7). Clearly, further fine mapping is necessary to identify the causal variant that exerts opposing effects on AD and psoriasis, but we speculate that *ANXA6* might be a switch-point differentiating AD from psoriasis that

Table 1. Loci Showing Genome-wide Significant Association with Either AD or Psoriasis Defined by All Three Methods of Comparative Analysis

Chr Band	Reference SNV Number(s)	Position (hg19)	Nearest Gene(s) or Transcript(s)	Effect Observed in GW Analyses	Estimated Odds Ratio (95% CI)		Previous Report(s) of Association at This Locus
					AD	Psoriasis	
1p31.3	rs77614545 (del)	67749581	retro-DNAJB6 and <i>IL23R</i> (MIM 607562)	psoriasis	0.99 (0.92–1.07)	1.21 (1.15–1.28)	psoriasis: 1p31.3 locus, <i>IL28RA</i> ^{21,24,40}
1q21.3 ^a	rs55879323	152168740	within <i>FLG-AS1</i>	opposing	0.76 (0.70–0.82)	1.05 (1.00–1.12)	AD and psoriasis: 1q21.3 locus, <i>HRNR</i> , <i>FLG</i> ; ⁴¹ <i>FLG</i> ; ^{20,42} <i>LCE3B</i> , <i>LCE3C</i> , ¹⁸ <i>LCE</i> gene cluster; ¹⁷ <i>LCE3D</i> ²⁴
	rs11205006, rs12144049	152440176, 152440910	RP1-91G5.3, <i>CRNN</i> (MIM 611312), <i>LCE5A</i> (MIM 612619)	AD	1.52 (1.41–1.64), 1.53 (1.42–1.64)	0.97 (0.92–1.03), 0.98 (0.92–1.03)	AD: 1q21.3 locus, <i>HRNR</i> , <i>FLG</i> ; ⁴¹ <i>FLG</i> ^{20,42}
	rs471144	152454255	<i>LCE5A</i> (MIM 612619)	AD	1.54 (1.37–1.73)	1.03 (0.94–1.14)	AD: 1q21.3 locus, <i>HRNR</i> , <i>FLG</i> ; ⁴¹ <i>FLG</i> ^{20,42}
	rs10888499	152532742	<i>LCE3E</i> (MIM 612617)	AD	1.49 (1.38–1.61)	0.98 (0.93–1.04)	AD: 1q21.3 locus, <i>HRNR</i> , <i>FLG</i> ; ⁴¹ <i>FLG</i> ^{20,42}
	rs4112788	152551276	<i>LCE3D</i> (MIM 612616)	psoriasis	0.97 (0.90–1.05)	1.22 (1.15–1.28)	psoriasis: <i>LCE</i> gene cluster; ¹⁷ <i>LCE3D</i> ²⁴
	rs1581803	152592281	<i>LCE3A</i> (MIM 612613)	psoriasis	0.97 (0.90–1.04)	1.22 (1.15–1.30)	psoriasis: <i>LCE</i> gene cluster ¹⁷
	rs77199844 (del)	152757094	<i>LCE1E</i> (MIM 612607)	[AD]	2.01 (1.72–2.35)	1.16 (1.01–1.33)	AD: 1q21.3 locus, <i>HRNR</i> , <i>FLG</i> ; ⁴¹ <i>FLG</i> ^{20,42} psoriasis: <i>LCE</i> gene cluster ¹⁷
	rs4363385	152989321	<i>SNORA31</i> , <i>SPRR3</i> (MIM 182271), <i>SPRR1B</i> (MIM 182266)	[opposing]	1.23 (1.15–1.32)	0.89 (0.85–0.94)	AD: <i>SPRR3</i> repeat number variant ⁴³
2p16.1	rs35741374	61072567	within lincRNA AC010733.4	psoriasis	1.09 (1.01–1.63)	1.20 (1.15–1.27)	psoriasis: <i>REL</i> ; ²⁴ <i>NR</i> ²³
2q31.2	rs62176107	179300971	exonic <i>PRKRA</i> and within miRNA548n and AC009948.5	opposing	0.55 (0.46–0.65)	1.42 (1.32–1.53)	–
5q31.1 ^a	rs1295686	131952222	intronic <i>IL13</i> (MIM 147683) and within AC004041.2	[opposing]	1.27 (1.17–1.38)	0.88 (0.82–0.94)	AD and psoriasis: <i>IL13</i> ; ²¹ <i>KIF3A</i> , <i>IL13</i> ; ²² <i>KIF3A</i> , <i>IL4</i> , <i>IL13-RAD50</i> ; ¹⁰ multiple effect locus <i>RAD50/IL13</i> ; ²⁰ <i>C5orf56</i>
	rs6596086	131995843	intronic <i>RAD50</i> (MIM 604040)	opposing	1.30 (1.20–1.41)	0.85 (0.8–0.91)	AD and psoriasis: <i>IL13-RAD50</i> ; ¹⁰ multiple effect locus <i>RAD50/IL13</i> ²⁰
5q33.1	rs17728338	150478318	<i>ANXA6</i> (MIM 114070)	opposing	0.70 (0.59–0.84)	1.77 (1.61–1.95)	psoriasis: <i>TNIP1</i> ^{21,42}
5q33.3	rs10515778, rs7715173, rs7719425 ^b	158658012, 158664631, 158670938	within CTB-11122.1	psoriasis	1.07 (0.98–1.17)	1.29 (1.21–1.38)	psoriasis: 5q33.3 locus, <i>IL12B</i> ^{17,21,24,44} ; AD: <i>PTTG1</i> ⁴²
	rs11135056, rs4921442 ^b	158687281, 158694100	intronic <i>UBLCP1</i> (MIM 609867)	psoriasis	0.97 (0.89–1.05)	1.45 (1.35–1.56)	
	rs2546890	158759900	within AC008697.1	psoriasis	1.01 (0.94–1.06)	1.39 (1.32–1.47)	
	rs5872599 (indel)	158859989	lincRNA AC008703.1, <i>IL12B</i> (MIM 161561)	psoriasis	0.82 (0.73–0.93)	1.54 (1.45–1.64)	
6q21	rs9487605	111582885	intronic <i>KIAA1919</i>	psoriasis	1.06 (0.98–1.14)	1.27 (1.20–1.35)	–
	rs240993	111673714	intronic <i>REV3L</i> (MIM 602776)	psoriasis	1.05 (0.97–1.13)	1.29 (1.22–1.36)	–
	rs9481169	111929862	<i>TRAF3IP2</i> (MIM 607043)	psoriasis	0.98 (0.86–1.11)	1.58 (1.45–1.72)	psoriasis and psoriatic arthritis: <i>TRAF3IP2</i> ^{24,44,45}

(Continued on next page)

Table 1. Continued		Reference SNV Number(s)	Position (hg19)	Nearest Gene(s) or Transcript(s)	Effect Observed in GW Analyses	Estimated Odds Ratio (95% CI)		Previous Report(s) of Association at This Locus
Chr Band						AD	Psoriasis	
6q23.2		rs643177, rs582757 ^b	138195693, 138197824	TNFAIP3 (MIM 610669)	psoriasis	1.05 (0.97–1.14)	1.27 (1.20–1.34)	psoriasis: TNFAIP3 ^{21,24}
11q13.5		rs2212434, rs7126418 ^b	76281593, 76292573	c11orf30 (MIM 608574)	AD	1.29 (1.21–1.39)	1.05 (1.00–1.11)	AD: c11orf30-LRRC32; ^{10,41} c11orf30; ²² 11q13 locus ²⁰
12q13.3		rs36207871 (del)	56684496	intronic CS (MIM 118950)	psoriasis	0.94 (0.83–1.06)	1.47 (1.33–1.67)	psoriasis: 12q13.3 locus, IL23A, STAT2; ²¹ IL23A ²⁴
		rs11575234	56744276	intronic STAT2 (MIM 600556)	psoriasis	0.90 (0.79–1.02)	1.47 (1.32–1.64)	psoriasis: STAT2 ²¹

Genome-wide significance is defined as CCMA $T_{max} > 6$ and MANTRA BFD $p < 10^{-8}$; genes and transcripts identified from UCSC Genome Browser Human Feb. 2009 (GRCh37/hg19) Assembly accessed 21 March 2014; this variant of FLG-AS1 extends across HRHR and FLG; RP1-91G5.3 extends across CRNN; AC004041.2 extends across RAD50 and IL13; CTB-11122.1 overlaps RNFI45.

^a1q21.3 and 5q31.1 were further investigated via stepwise conditional analysis (the results are shown in Table 2); square brackets indicate results from the univariate analysis that were subsequently accounted for by other nearby variants when examined by stepwise conditional analysis.

^bMultiple SNVs are assigned to the same LD block but odds ratios and 95% CI are presented only for the first SNV.

reflects the importance of calcium-dependent effects in keratinocyte differentiation.

Opposing effect loci were also identified within regions characterized by complex patterns of LD within the EDC (Figure S4), the cytokine cluster on 5q31.1 (Figures S4 and S5), and the MHC. These regions were therefore investigated further via conditional analysis.

Stepwise Conditional Analysis within 1q21.3 and 5q31.1 Identifies Opposing and Disease-Specific Risk Variants

Coverage of the EDC was achieved via GWAS data (Figure 3), whereas ImmunoChip data provided better coverage for the cytokine cluster on 5q31.1 (Figures 4 and S5).

Within 1q21.3 we identified seven LD blocks with disease-specific or opposing signals (Figure 3A). Stepwise conditional analysis on the four most prevalent *FLG*-null mutations and variants tagging the *LCE3B-LCE3C* deletion identified one AD-specific locus mapping to *FLG*, a psoriasis-specific locus mapping to *LCE3B-LCE3C*, and a locus with opposing effects on both diseases mapping to *RPTN* (MIM 613259)/*HRNR/FLG-AS1* (Figure 3B and Table 2). After conditioning on the four *FLG*-null mutations and the *LCE3B-LCE3C* deletion, the G allele of the lead MNM SNV rs12130219 decreases the risk for AD ($OR_{ADcond} = 0.812$, $p_{ADcond} = 0.0018$) and increases the risk for psoriasis ($OR_{PSOcond} = 1.119$, $p_{PSOcond} = 3.68 \times 10^{-4}$) (Table 2). Filaggrin, repetin, and hornerin are all members of the S100 fused-type protein family and each contribute to the cornified cell envelope, a functional component of the epidermal barrier. Both *FLG* and *HRNR* show reduced expression in AD^{51–53} whereas *RPTN* shows no significant difference (Table S7). In psoriasis *HRNR* expression can be downregulated⁵³ or upregulated,⁵⁴ *RPTN* expression might be upregulated, and *FLG* expression might be downregulated⁵⁵ or dysregulated¹⁵ (Table S7). The function of *FLG-AS1* (*FLG* antisense RNA1) is currently undefined, but its proximity to *FLG* and *HRNR* suggests a role in coordinating keratinocyte terminal differentiation. *FLG-AS1* expression is increased in psoriasis lesional compared with nonlesional skin, whereas in AD lesional skin, expression is reduced (Table S7). Together, our results confirm the role of the *LCE3B-LCE3C* deletion in psoriasis and support the presence of genetic risk mechanisms for AD within the EDC in addition to the predominant effect of *FLG*-null mutations, with opposing effects on psoriasis.

Conditional analysis at 5q31.1 revealed three independent loci specifically contributing to AD risk: *IL13* (MIM 147683, rs848, $OR_{ADfull} = 1.12$, $p = 0.0204$), *KIF3A* (MIM 604683, rs 2299009, $OR_{ADfull} = 1.16$, $p = 4.1 \times 10^{-4}$), and *SLC22A4* (MIM 604190)/*C5orf56* (rs74458173, $OR_{ADfull} = 1.57$, $p = 2.0 \times 10^{-4}$) (Figure 4A, Table 2). None of these loci showed significant effects on psoriasis. However, a fourth independent locus has opposing effects on AD and psoriasis. The most highly significant variant maps

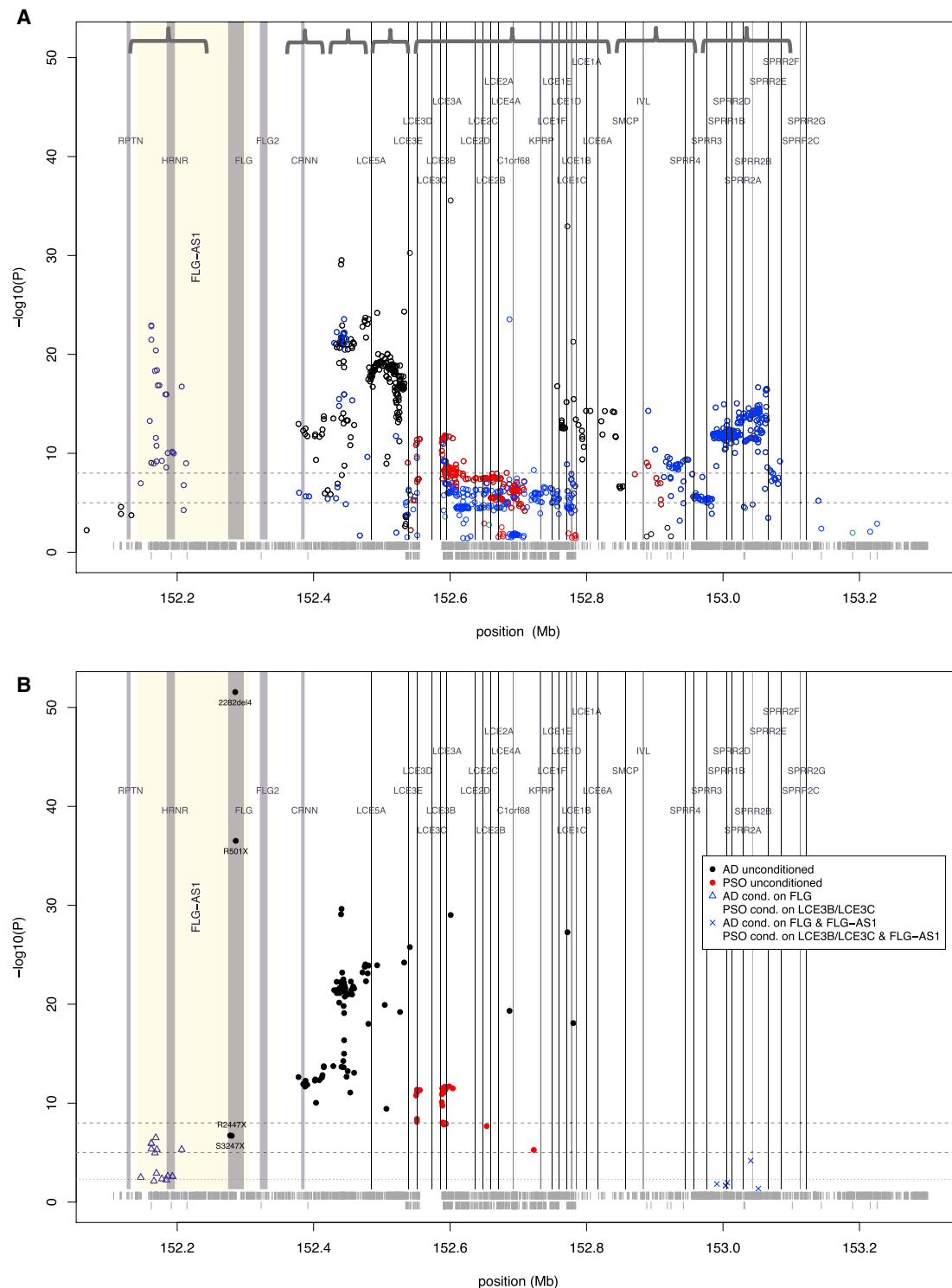


Figure 3. Regional Association within the Epidermal Differentiation Complex at 1q21.3

(A) Multinomial regression model with GWAS and ImmunoChip data. Seven blocks of linkage disequilibrium are indicated by curly brackets; black circles indicate AD-specific association, red circles indicate a psoriasis-specific association, blue circles represent opposing effects in AD and psoriasis, and green circles indicate shared effects. Vertical lines have been drawn to mark the positions of known genes and transcripts (identified from UCSC Genome Browser, GRCh37/hg19 accessed Feb. 2009) and the horizontal dotted lines indicate thresholds of suggestive and genome-wide significance ($p = 10^{-5}$ and 10^{-8}). The horizontal gray bands at the bottom indicate the coverage of the region by GWAS SNVs (upper row) and ImmunoChip SNVs (lower row).

(B) Conditional regional association plot of stepwise logistic regression using GWAS and ImmunoChip data. The different colored symbols indicate association results after each step of analysis, as follows. Unconditioned results are shown by black dots to indicate association with AD and red dots to indicate association with psoriasis; blue triangles and blue crosses represent results after conditioning on the known disease-associated variants, *FLG* in AD and *LCE3B-LCE3C* deletion in psoriasis; SNVs indicated by the same symbol are in LD

(legend continued on next page)

to *RAD50* (MIM 604040, rs6596086, $OR_{ADfull} = 1.17$, $OR_{Psofull} = 0.88$, $p = 6.3 \times 10^{-7}$); this variant is associated with increased risk of AD but is protective against psoriasis (Figure 4B, Table 2).

Analysis of the MHC Confirms Multiple Psoriasis-Risk Loci and Identifies Opposing Effects

In the extended HLA region, we took forward 23,479 SNVs with $T_{max} > 4.7$ or $BFDp < 0.05$ ($PO = 1/99$) and 75 variables representing the classical HLA alleles obtained from HLA imputation for multinomial modeling, of which 18,515 SNVs were classified as specific to psoriasis by CCMA and MNM. To reduce the data set for post hoc analysis, we considered only SNVs with effect classification in the same direction by CCMA and MNM, meeting the $p < 10^{-5}$ threshold in MNM. Within the psoriasis-specific markers, we excluded all tagging SNVs ($r^2 > 0.8$ with the lead SNV), resulting in 1,503 SNVs, including those previously reported for AD.²⁰

The strongest and most significant association was observed for psoriasis, a variant (rs111576655 $OR_{Psofull} = 3.32$, $p = 3.2 \times 10^{-65}$) tagging the well-known psoriasis-risk allele HLA-C*06:02 ($OR_{Psofull} = 3.59$, $p = 8.7 \times 10^{-154}$). Conditional analysis revealed two additional independent loci contributing to psoriasis risk at *MICA* (MIM 600169, rs201374403, $OR_{Psofull} = 1.65$, $p = 1.0 \times 10^{-26}$) and *HLA-A* (MIM 142800, rs113573479, $OR_{Psofull} = 1.41$, $p = 2.7 \times 10^{-17}$), as well as two loci with opposing effects at *HLA-C* (MIM 142840, rs1793889, $OR_{ADfull} = 0.6$, $OR_{Psofull} = 1.18$, $p = 1.1 \times 10^{-9}$) and *HLA-DRB1* (MIM 142857, rs28383201, $OR_{ADfull} = 0.61$, $OR_{Psofull} = 1.18$, $p = 6.5 \times 10^{-9}$) (Figure 5, Table 3). Conditional analysis with imputed classical alleles identified five independent HLA-class I alleles contributing to psoriasis risk in addition to HLA-C*06:02 and two alleles with opposing effects: HLA-C*03:03 ($OR_{ADfull} = 0.71$, $OR_{Psofull} = 1.27$, $p = 2.3 \times 10^{-5}$) and HLA-DQA1*02:01 ($OR_{ADfull} = 0.64$, $OR_{Psofull} = 1.09$, $p = 6.0 \times 10^{-8}$; $r^2 = 0.405$ with rs28383201) (Table 3).

Ontology and Network Analysis of Genes Indicate Effects in the Skin Barrier and Immune Response

Genes implicated from genome-wide and conditional analyses (identified from Tables 1, 2, and 3) were investigated via predicted protein network and gene ontology (GO) analysis. The results are summarized in Figure S6. The GO term “keratinocyte differentiation” (GO:0030216) is enriched in genes implicated in AD and psoriasis risk (FDR $p = 4.3 \times 10^{-4}$ in AD; $p = 6.9 \times 10^{-4}$ in psoriasis; and $p = 2.7 \times 10^{-3}$ in opposing effects). The GO term “response to interferon-gamma” (GO:0034341) is also significantly enriched in psoriasis (FDR $p = 1.9 \times 10^{-3}$).

Discussion

This genome-wide comparative analysis confirms a high degree of genomic coincidence between AD and psoriasis, suggesting that common molecular mechanisms are involved. This agrees with the central role of epidermal barrier defects and T-cell-dominated inflammation in both diseases.⁴⁸ Within the six regions of colocalization, we demonstrate coassociated and independent disease-specific loci. Of note, all coassociated loci display opposing (antagonistic) effects on AD and psoriasis, in agreement with the epidemiological observations of lower-than-expected coincidence between these diseases in the population.⁵ Within these loci, specific variants including chromosome 2q31.2 (rs62176107), chromosome 5q33.1 (rs17728338), and within *RAD50* on chromosome 5q33.3 (rs6596086) demonstrate opposing effects on risk of AD and psoriasis. This raises the intriguing possibility that the same biological mechanisms might act differentially on AD versus psoriasis. However, our current data cannot distinguish this specific opposing mechanism from the possibility that each lead variant is in LD with other variants having opposing effects in each disease.

The majority of the opposing effect loci are implicated in pathways related to adaptive immunological functions, which potentially mirrors the polarized immune mechanisms.⁶ It might further be speculated whether the presence of multiple opposing alleles reflects balancing selection as a response to heterogeneity in environmental pressures. Balancing selection is particularly common within the extended MHC region and has been proposed as a potential explanation for antagonistic effects at multiple loci in different autoimmune diseases.⁵⁶

Two of the loci displaying opposing effects (*ANXA6/TNIP1* and *PRKRA*) have not previously been reported in association with psoriasis and/or AD. Formal external validation is limited by the requirement for additional independent, population-matched GWAS data for AD and psoriasis, but data available from RNA sequencing and microarray analyses provide some support for the differential expression of *ANXA6/TNIP1* and *PRKRA* in AD and psoriasis, relative to normal or uninvolved skin. The lead variant within *PRKRA* might mediate opposing effects in AD and psoriasis via miRNA processing and/or cellular response to environmental stress, and we hypothesize that this reflects the striking differential susceptibility to viral and bacterial skin infections observed in AD and psoriasis. The opposing effect of variation in *ANXA6* suggests a role for calcium-dependent effects in defining patterns of skin inflammation.

On chromosome 1q21.3, apart from well-established AD-associated *FLG* mutations and psoriasis-associated

with the lead SNV of each stepwise conditional analysis (defined as $r^2 \geq 0.5$). Vertical lines are drawn to mark the positions of known genes and transcripts (identified from the UCSC Genome Browser GRCh37/hg19 accessed Feb. 2009), and horizontal dotted lines indicate significance thresholds of $p = 0.005$, 10^{-5} , and 10^{-8} . The horizontal gray bands at the bottom indicate the coverage of the region by GWAS SNVs (upper row) and ImmunoChip SNVs (lower row).

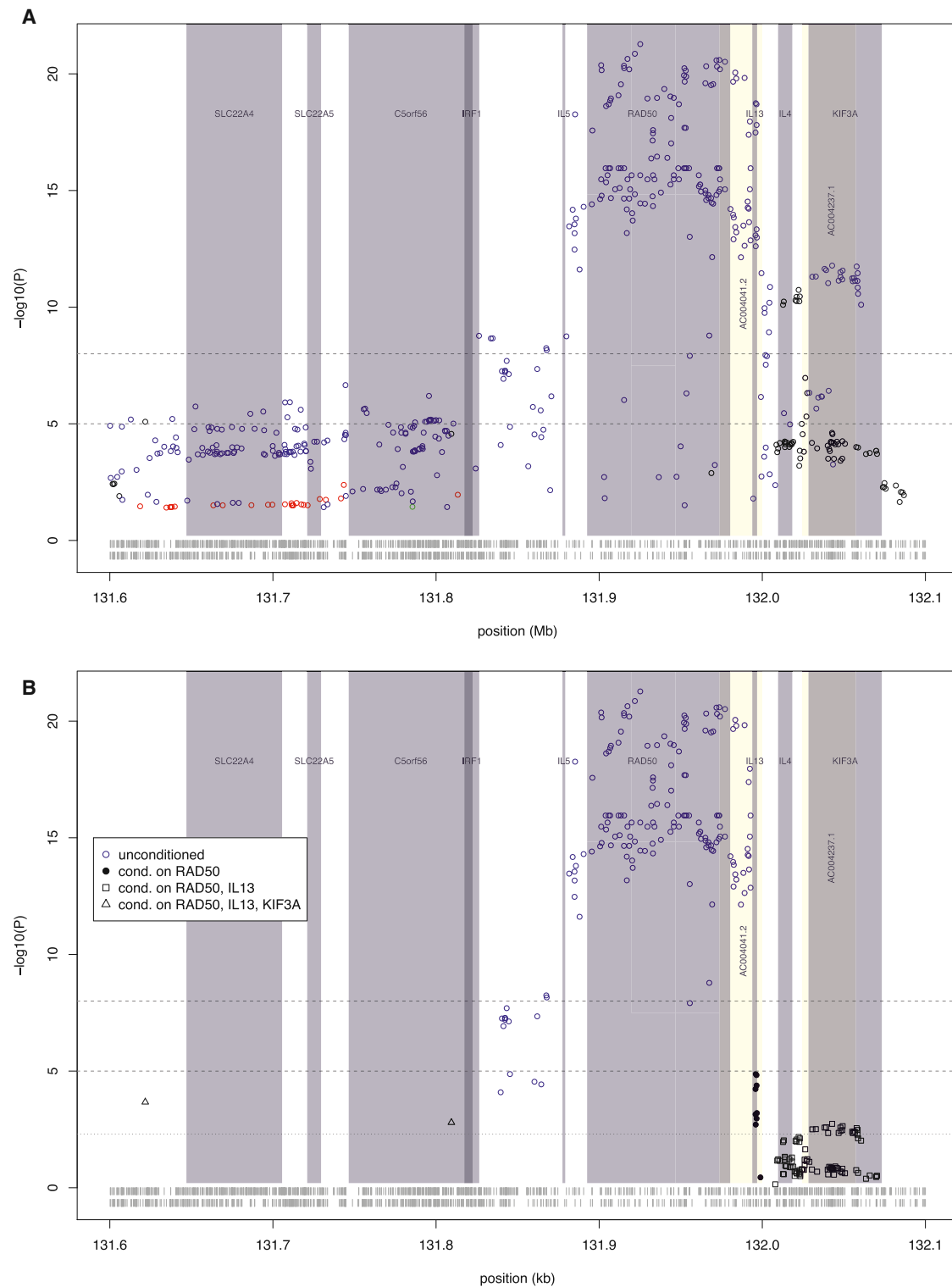


Figure 4. Regional Association within the Cytokine Cluster at 5q31.1

(A) Multinomial regression model with GWAS and ImmunoChip data. Black circles indicate AD-specific association, red circles indicate psoriasis-specific association, blue circles represent opposing effects in AD and psoriasis, and green circles indicate shared effects. Vertical gray shading marks the positions of known genes (identified from the UCSC Genome Browser GRCh37/hg19 accessed Feb. 2009), and horizontal dotted lines indicate suggestive and genome-wide significance thresholds ($p = 10^{-5}$ and 10^{-8} , respectively); results are shown for SNVs in LD with the lead SNV (defined as $r^2 \geq 0.5$). The horizontal bands at the bottom indicate the coverage of the region by GWAS SNVs (upper row) and ImmunoChip SNVs (lower row).

(B) Conditional regional association plot of the EDC by multinomial regression of GWAS and ImmunoChip data. Different symbols indicate association results after each step of analysis, as follows. Unconditioned results are shown by blue circles representing opposing effects in AD and psoriasis; black dots show AD-specific association results after conditioning on the lead SNV in *RAD50* (a gene reported to

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deletion of *LCE3B-LCE3C*, *FLG-AS1* is a plausible candidate to mediate differential AD/psoriasis risk via the network of regulatory elements coordinating gene expression.⁵⁷ Natural antisense transcripts contribute to gene regulation via a variety of transcriptional and posttranscriptional mechanisms⁵⁸ and include effects on human epidermal differentiation.⁵⁹ The proximity of *FLG-AS1* to *FLG* and *HRNR*, combined with data showing coordinated differential expression of these genes, supports a role in control of keratinocyte terminal differentiation.

On chromosome 5q31.1, antagonistic signals for AD and psoriasis have previously been attributed to *IL13*.^{10,11,21} We here show that *IL13* polymorphisms specifically influence AD risk, whereas opposing signals map to *RAD50*. The Rad50 protein, a component of the MRN complex (Mre11, Rad50, and Nbs1), is involved in DNA double-strand break repair but has no known function directly related to AD or psoriasis. However, *RAD50* mRNA shows significantly increased expression in psoriasis lesional skin and a trend to reduced expression in AD lesional skin (Table S7). Of note, *RAD50* is located in the center of the Th2-cytokine cluster and its 3' end is part of a locus control region regulating expression of these cytokine genes.⁶⁰ AD and psoriasis represent opposing extremes of Th2 cell dysregulation, and therefore we hypothesize that *RAD50* polymorphisms might exert opposing effects on AD and psoriasis through variation in DNA repair resulting in a differential skew in Th2 cell response.

Our dissection of the MHC locus confirms the presence of multiple independent psoriasis-risk loci. Markers tagging HLA-Cw*0602 generate the strongest effects, which is in line with previous reports.^{17,21,24,44,61} CD8⁺ T cells are increased in the epidermis of lesional psoriatic skin, and the association of psoriasis susceptibility primarily with class I HLA alleles might reflect the critical role of psoriasis-associated (auto-)antigen presentation to pathogenic CD8⁺ T cells.⁶² CD8⁺ T cells are also increased in the epidermis of AD skin, but with strikingly different cytokine profiles compared to psoriasis.⁶³ The opposing effects of class II HLA alleles in AD and psoriasis might represent the differential responses to pathogenic and allergenic peptides presented to CD4⁺ T cells.⁶⁴ GWASs in AD by univariate and multivariate models have reported association signals in the MHC class I and II regions^{22,65} and two specific HLA class II haplotypes, HLA-DRB1*0701 (a protective effect) and HLA-B*4402 (a risk effect).²⁰ Our analysis confirms the association of classical HLA class II alleles with AD, but in the conditional analysis, only HLA-DQA1*02:01 remained, showing a significant protective effect

on AD and a significant opposing effect on psoriasis. A further opposing locus mapped to HLA-C*03:03 (Table 3).

The reported observation of AD occurring within the offspring of parents with psoriasis⁸ is not supported by our findings, and the observation that both Th1-cell-dominated autoimmune and Th2-cell-dominated allergic diseases can show aggregation within families⁷ also presents a discrepancy with our analyses. It is possible that there are shared risk loci for AD and psoriasis that were not detected in our current study because of lack of power, if the shared effect is not strong; alternatively, there might be hereditary risk factors associated with predisposition to any chronic inflammatory (auto-)immune disease. It is also possible that diagnostic misclassification occurs, particularly in pediatric cases, where the clinical signs of psoriasis are more difficult to distinguish from AD than is the case in adult disease,⁶⁶ or by recall bias for disease in parents.

It is interesting to estimate the extent to which our findings can explain the mutual exclusivity of AD and psoriasis, but an accurate assessment is hindered by the lack of published data on the proportion of AD and psoriasis cases where the diseases do and do not co-occur. Henseler et al. report a 25-fold lower prevalence of AD occurring in psoriasis cases⁵ and assuming a prevalence of 10% and 2% for AD and psoriasis, respectively,^{3,4} we estimate that the effects at the six opposing loci listed in Tables 1, 2, and 3 would result in a reduction in prevalence of AD from 10% to 8% within the group of individuals with psoriasis. This 2% reduction contrasts with the 25-fold reduction reported by Henseler et al.,⁵ which is equivalent to a reduction of 9.6%, from 10% in the population to 0.4%. Our results have therefore explained approximately 21% ($2/9.6 \times 100$) of the mutual exclusivity of AD and psoriasis.

Taken together, our comparative analyses of AD and psoriasis support a paradigm in which genetic factors determining keratinocyte differentiation and cutaneous barrier function have particularly strong effects on AD risk, whereas in psoriasis genetic factors influencing (auto-)antigen recognition are of paramount importance. Furthermore, multiple pleiotropic loci with antagonistic effects contribute to opposing mechanisms of adaptive immunity in both AD and psoriasis.

The meta-analysis-inspired methodology developed in the course of this study has demonstrated the power to leverage additional information from GWAS and high-density SNV data and to dissect cross-phenotype associations. AD and psoriasis are particularly well suited to the compare/contrast approach, but this methodology will

be associated with AD and psoriasis); black squares indicate the residual AD-specific association after conditioning on the lead SNVs in *RAD50* and *IL13* (genes reported to be associated with AD); and black triangles indicate the residual AD-specific association after additionally conditioning on the lead SNV in *KIF3A* (a gene reported to be associated with AD). SNVs indicated by the same symbol are in LD with the lead SNV of each stepwise conditional analysis (defined as $r^2 \geq 0.5$). Vertical gray shading marks the positions of known genes (identified from the UCSC Genome Browser GRCh37/hg19 accessed Feb. 2009), and horizontal dotted lines indicate significance thresholds of $p = 0.005$, 10^{-5} , and 10^{-8} ; results are shown for SNVs in LD with the lead SNV (defined as $r^2 \geq 0.5$). The horizontal bands at the bottom indicate the coverage of the region by GWAS SNVs (upper row) and ImmunoChip SNVs (lower row).

Table 2. Conditional Analysis of the 1q21.3 and 5q31.1 Regions Showing Disease-Specific and Opposing Risk Effects in AD and Psoriasis

Data Source	Effect	SNV	Pos (hg19)	Allele	Candidate Genes	AD		Psoriasis			AD		Psoriasis		
						OR (95% CI)	p	OR (95% CI)	p	P _{overall} ^a	OR (95% CI)	p	OR (95% CI)	p	P _{overall} ^a
Chromosome 1q21.3															
Unconditioned Analysis						Conditional Analysis ^b									
GWAS	Opposing	rs12130219	152162106	<u>G</u> /A	<i>FLG-AS1/RPTN/HRNR</i>	0.66 (0.60–0.73)	1.1 × 10 ^{−16}	1.15 (1.09–1.224)	4.0 × 10 ^{−6}	1.2 × 10 ^{−23}	0.812 (0.71–0.93)	0.0018	1.119 (1.05–1.19)	3.68 × 10 ^{−4}	2.4 × 10 ^{−6}
GWAS	AD	rs12144049	152440910	<u>C</u> /T	<i>FLG</i>	1.53 (1.42–1.64)	2.7 × 10 ^{−30}	0.98 (0.92–1.03)	0.4140	3.0 × 10 ^{−30}	–	–	–	–	–
GWAS	Psoriasis	rs1581803 ^c	152592281	<u>G</u> /T	<i>LCE3B/LCE3D</i>	0.97 (0.90–1.04)	0.4396	1.22 (1.16–1.30)	1.5 × 10 ^{−12}	1.6 × 10 ^{−12}	–	–	–	–	–
GWAS	Opposing	rs35722864	153040505	G/GA	<i>SPRR</i> cluster	0.81 (0.75–0.88)	1.0 × 10 ^{−7}	1.129 (1.07–1.20)	2.1 × 10 ^{−5}	4.8 × 10 ^{−13}	0.851 (0.71–0.93)	0.0019	1.074 (1.01–1.14)	0.0211	1.3 × 10 ^{−4}
Chromosome 5q31.1															
Conditional Models ^d						Full Model									
Ichip	Opposing	rs6596086	131952222	<u>C</u> /T	<i>RAD50</i>	1.31 (1.22–1.41)	1.7 × 10 ^{−13}	0.86 (0.80–0.92)	1.7 × 10 ^{−5}	5.7 × 10 ^{−21}	1.17 (1.07–1.28)	4.04 × 10 ^{−4}	0.88 (0.81–0.96)	0.0023	6.3 × 10 ^{−7}
Ichip	AD	rs848	131996500	<u>A</u> /C	<i>IL13</i>	1.20 (1.10–1.30)	5.6 × 10 ^{−5}	0.96 (0.89–1.04)	0.3375	4.14 × 10 ^{−5}	1.12 (1.02–1.23)	0.0197	0.96 (0.88–1.05)	0.3515	0.0204
Ichip	AD	rs2299009	132042813	<u>G</u> /T	<i>IL4/KIF3A</i>	1.14 (1.06–1.23)	7.9 × 10 ^{−4}	0.99 (0.92–1.06)	0.7392	0.0018	1.16 (1.07–1.25)	2.03 × 10 ^{−4}	0.99 (0.92–1.06)	0.6657	4.1 × 10 ^{−4}
Ichip	AD	rs74458173	131621731	<u>A</u> /C	<i>SLC22A4</i>	1.57 (1.26–1.96)	6.1 × 10 ^{−5}	1.02 (0.80–1.30)	0.8590	2.14 × 10 ^{−4}	1.57 (1.26–1.96)	5.71 × 10 ^{−5}	1.02 (0.80–1.30)	0.8683	2.0 × 10 ^{−4}

Full model incorporates the combined effects of independent SNVs identified by stepwise analyses.

^a P_{overall} represents the overall opposing signal calculated using the $T_{12\text{opposing}}$ statistic and derive the p value from the normal distribution.

^bConditional analysis of chr1q21.3 was conditioned on *FLG* for AD and *LCE3B/LCE3D* for psoriasis.

^crs1581803 tags the previously reported psoriasis SNV rs4112788 ($r^2 = 0.995$).

^dStepwise conditional analysis at chr5q31.1 was carried out using multinomial regression models and resulted in three additional signals for AD; this table shows only independent loci ($r^2 < 0.5$) and the SNV with the strongest association; the effect allele is underlined.

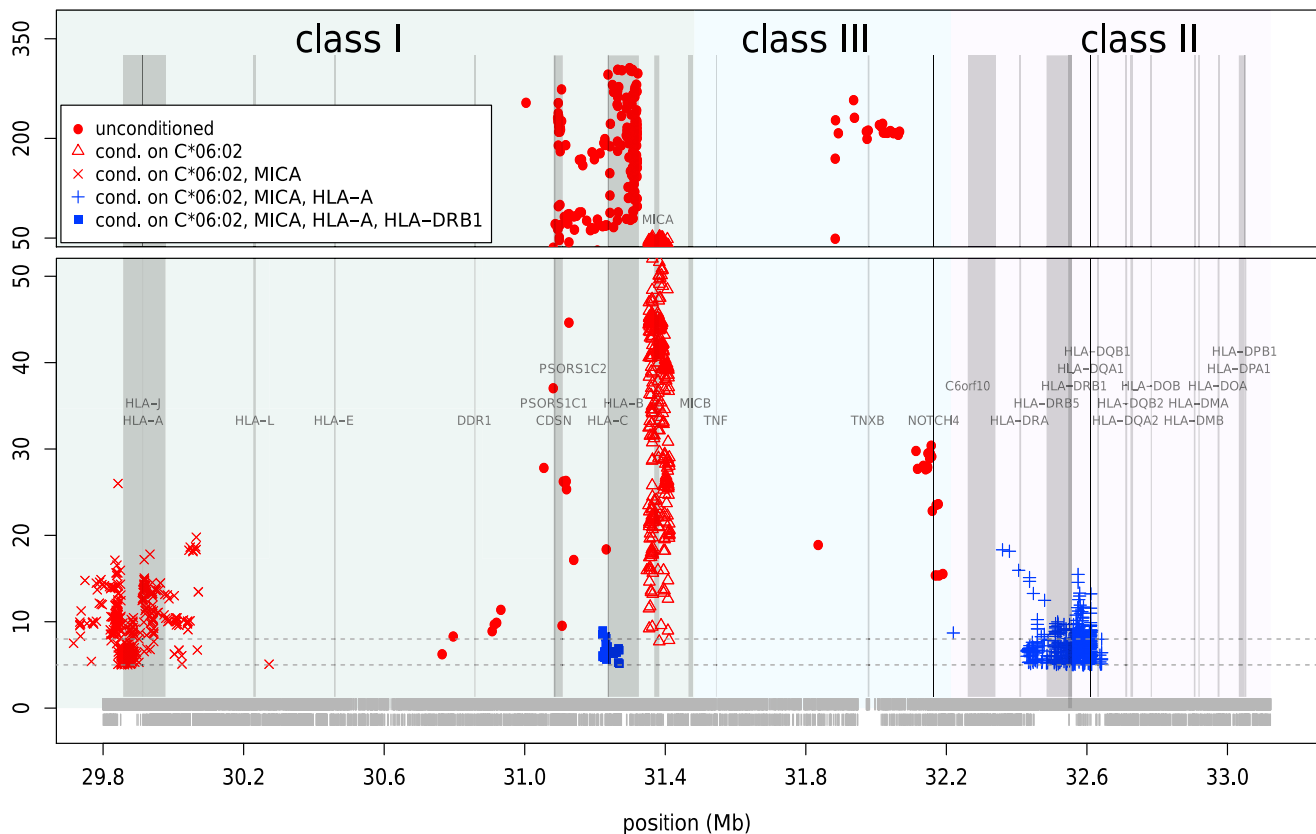


Figure 5. Conditional Regional Association within the Major Histocompatibility Complex at 6p21–22 via GWAS and ImmunoChip Data

Symbols indicate association results after each step of analysis, as follows. Unconditioned psoriasis-specific results are shown by red dots; red triangles show psoriasis-specific association results after conditioning on C*06:02 (known to be strongly associated with psoriasis); red \times s indicate psoriasis-specific association after conditioning on C*06:02 and MICA; blue +s indicate the association after conditioning on C*06:02, MICA, and HLA-A with opposing effects on AD and psoriasis; and blue squares indicate the residual association after conditioning on C*06:02, MICA, HLA-A, and HLA-DRB1 with opposing effects on AD and psoriasis. SNVs indicated by the same symbol are in LD with the lead SNV of each stepwise conditional analysis (defined as $r^2 \geq 0.5$). Vertical shading marks the positions of known genes (identified from the UCSC Genome Browser GRCh37/hg19 accessed Feb. 2009) and HLA classes; horizontal dotted lines indicate significance thresholds of $p = 10^{-5}$ and $p = 10^{-8}$; results are shown for SNVs in LD with the lead SNV (defined as $r^2 \geq 0.5$). The horizontal bands at the bottom indicate the coverage of the region by GWAS SNVs (upper row) and ImmunoChip SNVs (lower row).

be applicable to many other complex traits with overlapping and disease-specific phenotypic features. Characterizing shared and opposing molecular mechanisms across complex phenotypes will expand our understanding of biology and disease and will have implications for treatment and drug discovery.

Supplemental Data

Supplemental Data include Supplemental Consortia Information, six figures, and seven tables and can be found with this article online at <http://dx.doi.org/10.1016/j.ajhg.2014.12.004>.

Consortia

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Table 3. Conditional Analysis of the MHC Region on 6p21–22 Showing Psoriasis-Specific and Opposing Risk Effects in AD and Psoriasis

Data Source	Effect	SNV	Pos (hg19)	Allele	HLA Allele/ Candidate Genes	Conditional Models ^a					Full Model					
						AD		Psoriasis			P _{overall}	AD		Psoriasis		
						OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)		p	OR (95% CI)	p		
GWAS	PSO	rs111576655	31242731	<u>A</u> /T	C*06:02	0.84 (0.74–0.95)	0.0053	4.41 (4.10–4.74)	3.1×10^{-376}	9.8×10^{-380}	1.12 (0.81–1.54)	0.5071	3.32 (2.90–3.81)	2.3×10^{-69}	3.2×10^{-65}	
GWAS	PSO	rs201374403	31383754	<u>T</u> /TAG	MICA	0.78 (0.7–0.88)	6.4×10^{-5}	1.68 (1.56–1.8)	7.9×10^{-48}	2.2×10^{-53}	0.81 (0.67–0.96)	0.0174	1.65 (1.50–1.81)	1.8×10^{-25}	1.0×10^{-26}	
GWAS	PSO	rs113573479	29842444	<u>A</u> /G	HLA-A	0.89 (0.81–0.97)	0.0109	1.39 (1.30–1.49)	6.6×10^{-25}	1.0×10^{-26}	0.92 (0.81–1.04)	0.1948	1.41 (1.30–1.52)	2.8×10^{-17}	2.7×10^{-17}	
GWAS	opposing	rs28383201	32574869	<u>C</u> /G	HLA-DRB1	0.59 (0.51–0.68)	4.6×10^{-13}	1.15 (1.06–1.24)	4.5×10^{-4}	3.3×10^{-16}	0.61 (0.52–0.71)	3.4×10^{-10}	1.18 (1.08–1.28)	1.0×10^{-4}	6.5×10^{-14}	
GWAS	opposing	rs1793889	31222181	<u>A</u> /G	HLA-C	0.60 (0.50–0.73)	2.5×10^{-7}	1.18 (1.07–1.31)	0.0011	1.1×10^{-9}	0.60 (0.50–0.73)	2.5×10^{-7}	1.18 (1.07–1.31)	0.0011	1.1×10^{-9}	
Data Source	Effect	HLA Allele	HLA Allele Frequency in Ps/AD/Controls	Conditional Models ^a					Full Model							
				AD		Psoriasis			P _{overall}	AD		Psoriasis				
				OR (95% CI)	p	OR (95% CI)	p	OR (95% CI)		p	OR (95% CI)	p				
GWAS	PSO	C*06:02	0.271/0.075/0.089	0.81 (0.71–0.91)	8.48×10^{-4}	4.28 (3.98–4.61)	2.9×10^{-362}	1.30×10^{-368}	0.97 (0.82–1.15)	0.7475	3.59 (3.26–3.95)	2.1×10^{-159}	8.7×10^{-154}			
GWAS	PSO	A*02:01	0.28/0.227/0.239	0.95 (0.88–1.03)	0.1793	1.32 (1.24–1.40)	4.1×10^{-20}	1.1×10^{-20}	0.99 (0.90–1.08)	0.7739	1.32 (1.24–1.41)	1.8×10^{-17}	1.1×10^{-16}			
GWAS	PSO	B*57:01	0.13/0.017/0.032	0.61 (0.46–0.80)	3.83×10^{-4}	1.63 (1.44–1.84)	8.0×10^{-15}	5.8×10^{-20}	0.60 (0.42–0.85)	0.0039	1.58 (1.38–1.81)	3.8×10^{-11}	1.1×10^{-13}			
GWAS	PSO	C*12:03	0.048/0.044/0.038	0.87 (0.74–1.03)	0.1169	1.74 (1.54–2.00)	5.2×10^{-18}	3.5×10^{-19}	0.86 (0.72–1.04)	0.1295	1.85 (1.62–2.11)	7.5×10^{-20}	1.8×10^{-20}			
GWAS	PSO	B*27:05	0.032/0.02/0.025	0.80 (0.63–1.01)	0.0619	1.59 (1.37–1.86)	2.9×10^{-9}	3.2×10^{-10}	0.74 (0.55–1.00)	0.0499	1.50 (1.28–1.76)	7.3×10^{-7}	1.8×10^{-7}			
GWAS	PSO	A*01:01	0.221/0.154/0.166	1.03 (0.94–1.13)	0.5475	1.25 (1.17–1.35)	2.3×10^{-10}	1.7×10^{-9}	1.08 (0.96–1.21)	0.2013	1.23 (1.14–1.33)	6.8×10^{-8}	4.0×10^{-7}			
GWAS	opposing	C*03:03	0.037/0.025/0.038	0.66 (0.54–0.81)	9.71×10^{-5}	1.30 (1.13–1.49)	2.5×10^{-4}	5.9×10^{-8}	0.71 (0.56–0.90)	0.0040	1.27 (1.10–1.47)	0.0011	2.3×10^{-5}			
GWAS	opposing	DQA1*02:01	0.186/0.055/0.098	0.64 (0.54–0.76)	3.44×10^{-7}	1.09 (1.01–1.19)	0.0385	6.0×10^{-8}	0.64 (0.54–0.76)	3.4×10^{-7}	1.09 (1.01–1.19)	0.0385	6.0×10^{-8}			

Effect allele is underlined. Abbreviations are as follows: PSO, psoriasis; AD, atopic dermatitis. Table shows only independent loci ($r^2 < 0.5$) and the SNV with the strongest association.

^aStepwise conditional analysis was carried out with multinomial regression models and resulted in three psoriasis-specific and two opposing signals.

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Web Resources

The URLs for data presented herein are as follows:

1000 Genomes, <http://browser.1000genomes.org>
 Ensembl Genome Browser, <http://www.ensembl.org/index.html>
 Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org/>
 PLINK, <http://pngu.mgh.harvard.edu/~purcell/plink/>
 R statistical software, <http://www.r-project.org/>
 RefSeq, <http://www.ncbi.nlm.nih.gov/RefSeq>
 STRING 9.1, <http://www.string-db.org/>

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